STEROID METABOLISM IN ESSENTIAL HYPERTENSION

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STEROID METABOLISM

IN

ESSENTIAL HYPERTENSION

by

ARUN CHANDRA DEY

A THESIS

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ABSTRACT

Alterations of steroid metabolism in hypertension have been investigated with male and female normal and essential hypertensive subjects. Three groups of urinary metabolites have been quantitated: 1. androsterone, etiocholanolone and dehydroepiandrosterone (11-deoxy-17-ketosteroids, $C_{19}O_2$-KS), 2. 11-keto and 11β-hydroxy androsterone and 11-keto and 11β-hydroxy etiocholanolone (11-oxy-17-ketosteroids, $C_{19}O_3$-KS), and 3. 17-ketogenic steroids (KGS) oxidised to etiocholanolone, 11β-hydroxy androsterone and 11β-hydroxy etiocholanolone. The urinary steroids were hydrolysed enzymatically, fractionated on silica gel columns and estimated as trimethyl silyl ether derivatives by gas-liquid chromatography.

The following statistically significant differences were found in the excretion of metabolites by normal and hypertensive subjects.

1. $C_{19}O_2$-17KS fraction: the excretion of etiocholanolone glucuronide and dehydroepiandrosterone sulfate was markedly reduced in the hypertensive group (P 0.001), and the ratio of the 5α/5β-reduced metabolites was higher (P 0.001). The latter suggested a relative deficiency of 5β-reductase activity in the hypertensive group.

2. $C_{19}O_3$-17KS fraction: the excretion of the 5α-reduced metabolites was higher (P 0.01) in the hypertensive group, and the 5α/5β ratio was also increased (P 0.01), due to the increased 5α-compounds. No significant reduction was found for the 5β-reduced metabolites.

3. KGS fraction: the excretion of both 5α- and 5β- reduced compounds was higher (P 0.05) in the hypertensive group, but the 5β-compounds predominated in both normal and hypertensive groups. An increased excretion of 5β-reduced metabolites may suggest that the 5β-reductase activity is more active in the metabolism of the 17-ketogenic steroids than the $C_{19}O_2$-17KS.

The influence of the "11-oxy" function and corticosteroid "Side chain" on the relative 5α- and 5β- reductase enzyme activities and the influence of blood pressure has been discussed.

Comparison of the proportions of reduced metabolites in the urine of normal and essential hypertensive subjects indicates that they are relatively unaffected by age, sex or intensity of blood pressure.

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Systematic and trivial terminology and abbreviations

(1) Steroids

The following trivial and systematic names for steroids are used as given by the IUPAC (1969)

Aldo:	Aldosterone:	4-pregnen-18-al-11β,21-diol-3,20-dione (11-18)-lactol
	Androstenediol:	5-androsten-3β-17β-diol
	Adrenosterone:	4-androsten-3,11,17-trione
A:	Androsterone:	5α-androstan-3α-ol-17-one
		5α-androst-16-ene-3α-ol
Δ^4-A:	Androstenedione:	4-androstene-3,17-dione
		5-androsten-3α-ol-17-one
Chol:	Cholesterol:	5-cholesten-3β-ol
F:	Cortisol:	4-pregnan-11β,17α,21-triol-3,20-dione
	α-cortol:	5β-pregnan-3α,11β,17α,20α,21-pentol
	β-cortol:	5β-pregnan-3α,11β,17α,20β,21-pentol
	α-cortolone:	5β-pregnan-3α,17α,20α,21-tetrol-11-one
	β-cortolone:	5β-pregnan-3α,17α,20β,21-tetrol-11-one
DHEA:	Dehydroepiandrosterone:	5-androsten-3β-ol-17-one
DT:	Dihydrotestosterone:	5α-androstan-17β-ol-3-one
	Dihydroandrosterone:	5α-androstan-3α,17β-diol
	Epiandrosterone: Isoandrosterone:	5α-androstan-3β-ol-17-one
E:	Etiocholanolone:	5β-androstan-3α-ol-17-one

E_2:	Estradiol:	1,3,5 (10)-estratrien-3,17β-diol
E_1:	Estrone:	1,3,5 (10)-estratrien-3-ol-17-one
HA:	11β-hydroxyandrosterone:	5α-androstan-3α,11β-diol-17-one
HE:	11β-hydroxyetiocholanolone:	5β-androstan-3α,11β-diol-17-one
	11β-hydroxyadrenosterone:	4-androsten-11β-ol-3,17-dione
	11-hydroxyisoandrosterone:	5α-androstan-3β,11β-diol-17-one
	3β-hydroxyetiocholanolone:	5β-androstan-3β-ol-17-one
KA:	11-ketoandrosterone:	5α-androstan-3α-ol-11,17-dione
KE:	11-ketoetiocholanolone:	5β-androstan-3α-ol-11,17-dione
	11-ketoisoandrosterone:	5α-androstan-3β-ol-11,17-dione
PD:	Pregnanediol:	5β-pregnane-3α,20α-diol
PT:	Pregnanetriol:	5β-pregnane-3α,17α,20α-triol
	Testosterone:	4-androsten-17β-ol-3-one
THF:	Tetrahydrocortisol:	5β-pregnan-3α,11β,17α,21-tetrol-20-one
Allo-THF:	allo-tetrahydrocortisol:	5α-pregnan-3α,11β,17α,21-tetrol-20-one
	Tetrahydrocortisone:	5β-pregnan-3α,17α,21-triol-11,20-dione
THS:	Tetrahydro-11-deoxycortisol:	5β-pregnan-3α,17α,21-triol-20-one
17-KS:	17-ketosteroids	
17KGS:	17-ketogenicsteroids	
17-OHCS:	17-hydroxycorticosteroids	
$C_{19}O_2$-KS:	11-deoxy-17-ketosteroids	
$C_{19}O_3$-KS:	11-oxy-17-ketosteroids	
(2)	Solvents	
MeOH:	methanol:	methyl alcohol
EtOH:	ethanol:	ethyl alcohol
$MeCl_2$:	methylene chloride:	1,2-dichloro methane

$EtCl_2$:	ethylene chloride:	1,2-dichloro ethane
EtOAC:	ethyl acetate	

(3) Chromatographic terms

PC:	Paper chromatography	
TLC:	Thin layer chromatography	
GLC:	Gas liquid chromatography	
RT:	Retention time	
RRT:	Relative retention time	
HMDA:	Hexamethyl disilazane:	1,1,1,3,3,3-hexamethyl-disilazane
TMCS:	Trimethyl chlorosilane:	1,1,1-trimethyl-4-chlorosilane
TMSE:	Trimethyl silylether	
CMDMS:	Chloromethyl dimethyl silyl ether	

(4) Miscellaneous

N:	Normotensive	
H:	Hypertensive	
G:	Glucuronide	
S:	Sulfate	
DNB:	m-Dinitrobenzene	
TBS:	Toluene base scintillator	
DID:	Double isotope derivative	
BT:	Bluetetrazolium:	3,3-dimethoxy-4,4-biphenylene-bis-2, 5-diphenyl-2H-tetrazolium chloride
PPO:		2,5-diphenyloxazole
POPOP:		1,4-bis-2,5-phenyloxazolyl-benzene

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INTRODUCTION

Hypertension

The medical and socioeconomic importance of hypertensive disease may be realised from a recent statement that "a calculation based on careful estimates shows that over 8-10 million persons in the U.S.A. have significantly elevated blood pressure, and hypertensive vascular disease accounts for approximately 200,000 deaths per year" (Moser and Goldman, 1967).

The term hypertension, as commonly used, implies an elevation of systolic and diastolic blood pressures. Arteries are largely autonomous, and the normal artery tends to contract against a rising pressure, and to relax when that pressure falls. The mechanism of this autoregulation is still far from clear. The tension in the vessel wall varies with the radius of the vessel, as well as with the filling tension and may involve a myogenic factor (Byrom, 1969).

The dividing line between normal and high blood pressure is difficult to define. Evans and Rose (1971) have suggested that "in an operational sense, hypertension should be defined in terms of a blood pressure level above which investigation and treatment do more good than harm". Pickering (1970) holds the view that the terms "normotension" and "hypertension" were devised to help doctors to make a diagnosis. This critical dividing line will vary, of course, with age, sex, medical facilities available, follow up procedures and personal assessment of "good" and "harm". Considering these factors, it is not surprising that different levels of diastolic/systolic blood pressure have been defined to distinguish normotension from hypertension. Pickering (1970) quotes the following values as given by different workers: $\frac{140}{90}$, G.A. Perera (1948); $\frac{150}{90}$, C.B. Thomas (1952); and $\frac{180}{110}$, W. Evans, (1956).

High blood pressure is a sign, not a disease. It has a complex relationship with vascular disease, which is often symptomless until it is far advanced. There are several disease states in which hypertension may be a prominent sign. They include: glomerulonephritis, pyelonephritis, renal artery stenonis, coarctation of aorta, polycystic kidneys, desseminated lupus, eclamsia, brain tumor, poliomyelitis, acromegaly, primary hypothyroidism, pheochromocytoma, Cushing's syndrome and primary aldosteronism (Pickering, 1970; Laidlaw, 1973). In each of these situations, the actual mechanism by which the blood pressure is elevated may or may not be peculiar to that disease, but treatment of the disease usually cures the associated hypertension. However, in some 85-90% of the hypertensive population, there is no evident cause for the high blood pressure (Pickering, 1970). Such patients have been described as essential hypertensives, but some people prefer the term "primary hypertension" (Laidlaw, 1973). Essential hypertension is a symptomless disease at first, when an elevated blood pressure is the only positive finding. Urinalysis, intravenous pyelogram, blood and renal function tests reveal no abnormalities. However, the later consequences of hypertension may affect the target organs such as, brain, fundi, heart or kidney. Although no specific cause has been identified as responsible for the high blood pressure in essential hypertension, such factors as inheritance, age, and environmental conditions may be involved. However, the extent to which these factors are involved obviously varies considerably between individuals.

Any type of hypertension may rapidly become "malignant", or may remain in a relatively stable "benign" phase, as originally recognized by Volhard and Fahr (1914). This differentiation is based on the severity of the hypertension, but not on its kind. The malignant phase is characterised when hypertension is associated with grade iv changes in the optic fundi (Laidlaw, 1973), and though it may occur at any age in either sex, is most common at about 40 years of age and rarely develops after 60 (Pickering, 1970). The

casual diastolic pressure is nearly always over 130 mm Hg.

The more frequent benign phase occurs in all types of hypertension, but is more common when the onset occurs in middle aged or elderly subjects, and particularly in those with essential hypertension (Pickering, 1970). The arterial pressure during the benign phase is usually lower than in the malignant phase, and the patients in the early part of the phase are usually symptomless apart from the elevated blood pressure. Later cardiovascular complications may occur such as cardiac enlargement and heart failure, and occasionally lead to the malignant phase. But, whether such complications function as a precursor and contribute to the malignant phase is debatable.

The literature on the endocrine and non-endocrine aspects of hypertension is very extensive and a complete review of this subject is beyond the scope of this thesis. However, the endocrine aspects of hypertension will be reviewed as they relate to four categories of hypertension, namely: Renovascular, Catecholamine, Steroid and Essential (Bartter et al, 1970).

This will provide the background for the present investigation of steroid metabolism in essential hypertension.

Endocrine Aspects of Hypertension

1. The Renin-angiotensin system

The renin-angiotensin system holds a critical position in any discussion of hypertension. The kidney, in addition to having an excretory function, has what resembles an endocrine function in secreting an enzyme, renin, into the blood stream. This function was demonstrated by Goldblatt (1934) who reduced the blood supply to a dog's kidney by compression of the renal artery; a procedure which produced a persistant hypertension. It was initially assumed that the renin was directly responsible for the pressor function of the kidney, but subsequent experiments showed that renin exerted its action by producing a potent vasoconstrictor, angiotensin II.

Formation and storage of renin

Renin is a proteolytic enzyme that is synthesized in the juxtaglomerular apparatus of the kidney. This structure has two parts in the mammalian kidney (Faarup, 1965). One part, consisting of granular storage and secretory cells called juxtaglomerular cells (J.G. cells), is located near the afferent glomerular arteriole. The other part is termed the macula densa and consists of large columner cells located near the epithelium of the distal tubule.

Cook and Pickering (1958) have shown by microdissection of the kidney that renin is located in the J.G. cells; and that the relative abundance of the secretory granules in these cells closely parallel both the secretion and quantity of extractable renin (Tobian et al, 1959). Later, use of renin-antibodies provided further evidence that renin was localized in the granular J.G. cells (Hartroft, 1964). Recently Genest and co-workers have isolated a renin-like enzyme in the brain tissue of dogs which (a) is independent of kidney and plasman renin, (b) in presence of substrate forms angiotensin and (c) administration of aldosterone decreases its activity while progesterone increases it (Ganten et al, 1971).

Factors stimulating release of renin

Several stimuli are known to cause the release of renin from the kidney, as indicated below.

Sodium balance

A low intake of sodium increases and a high intake decreases the secretion of renin. Diuretics such as mercurials and thiazides, which cause a depletion of body sodium, also stimulate its secretion (de Champlain et al, 1965; Conn et al, 1965b), and it has been suggested that the sodium concentration in the renal tubule of the macula densa is involved in these situations (Vander & Miller, 1964; Von Schnerman et al, 1965). The role of extracellular cations on the release of renin has also been investigated. Increased plasma sodium and potassium inhibits while calcium stimulates the release of renin (Vander, 1970; Michelakis, 1971).

Arterial pressure

A decrease in arterial pressure increases the secretion of renin as can be demonstrated by constriction of the renal artery. The J.G. cells appear to be sensitive to the stretch in the arterial wall, and to act as baroreceptors. The increased blood pressure presumably "stretches" the granular cells and inhibits the release of renin, whereas a decreased pressure reduces the distention and enhances renin release (Tobian et al, 1958; Skinner et al, 1963; Lowe, 1964; Brown et al, 1965b).

Extra cellular fluid

The volume of the extra cellular fluid also seems to affect renin secretion, since the latter is stimulated by acute blood loss and depressed by hypertransfusion (Gross et al, 1964b). Mild hemorrhage also appears to activate the release of renin through the renal sympathetic nerves (Hodge et al, 1966; Bunag et al, 1966).

Sympathetic nerves and norepinephrine

The renin content of the denervated kidney has been shown to be greatly reduced as compared to normal kidneys (Taquini et al, 1964; Tobian et al, 1967). Infusion of norepinephrine directly into the renal artery also stimulates renin release from the kidney (Bunag et al, 1966; Udea et al, 1970). More recent studies have shown that circulating norepinephrine stimulates the J.G. cells and renin secretion, and indicate that the sympathetic nervous system may be involved through a β-receptor mechanism (Ganong, 1972; Tanigawa et al, 1972).

Formation of angiotensin I and II

After the release of renin from the kidney into the renal vein it acts upon a specific substrate, termed angiotensinogen, which is an α_2-globulin. Angiotensinogen is normally present in the blood and is synthesized in the liver. The renin acts specifically at the leucyl-leucine link of the substrate and splits off a decapeptide called angiotensin I, which is further broken down by a second enzyme, termed a converting enzyme, which is present in largest con-

centration in the lung. The product angiotensin II is formed by removal of two C-terminal amino acids, leucine and histidine, to give the octapeptide.

Vasopressor activity of angiotensin II

Angiotensin II is one of the most potent vasoactive substances known, being about 6-8 times as potent as norepinephrine (Page and Bumpus, 1961).

Infusion of angiotensin II into man causes an increase in the arterial pressure, a decreased pulse rate, heart beat and cardiac output, and a constriction of the pulmonary artery. Infusion of angiotensin II directly into the brachial artery of the human produces vasconstriction in the forearm muscle and hand (Page and Olmstead, 1961; Bock and Gross, 1961; Bono et al, 1963). Similar effects have been demonstrated in the cat (Barer, 1961) and the dog (Olmstead and Page, 1965; McCubbin et al, 1965). Recently it has been shown that infusion of angiotensin II at low rates into the vertebral arteries of anaesthetized dogs causes elevation of blood pressure (Ferrario et al, 1970; Severs et al, 1970). Administration of angiotensin II intraventricularly into the brain of male rats causes antidiuresis, loss of sodium and potassium (Severs et al, 1971). Others have reported that direct administration of angiotensin II into the brain leads to an increase in the release of acetylcholine in the cat (Elie and Panisset, 1970), vasopressin in the rat (Severs et al, 1970) and noradrenalin in the rabbit during sympathetic nerve stimulation (Schumann et al, 1970). However, the mechanism involving central nervous system and angiotensin II is not completely understood.

Destruction of angiotensin II

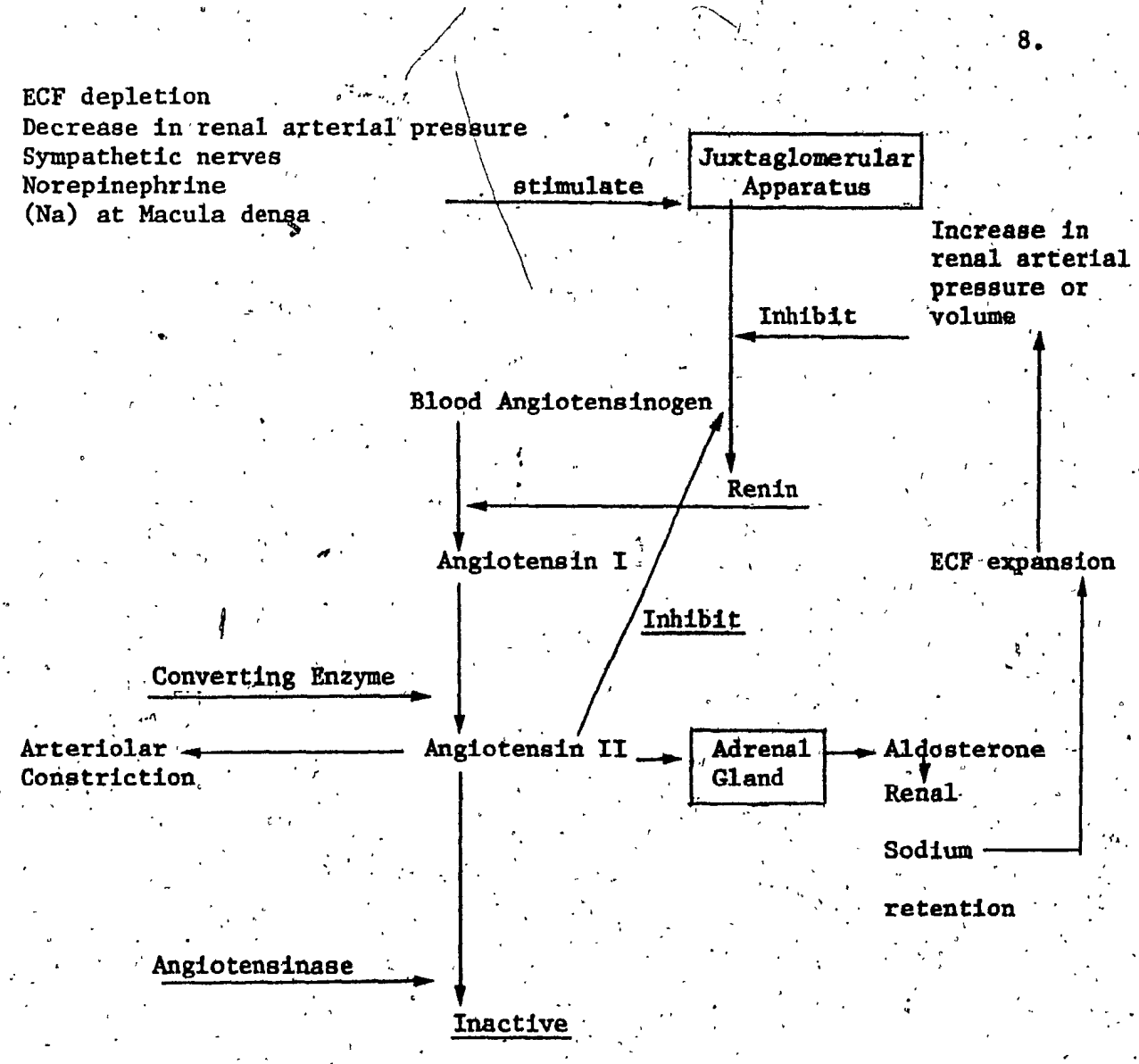
The biological activity of angiotensin II in the blood stream is limited since it is rapidly destroyed. It has also been observed that when renin is incubated in vitro with renin substrate for a prolonged period, angiotensin II disappears due to the action of a group of peptidases called angiotensinases (Page and Helmer, 1940). These enzymes are present in the

normal kidney, intestinal mucosa, plasma and red blood cells. Angiotensin II is also destroyed by trypsin, chymotrypsin and pepsin. (Pickering, 1970).

Action of angiotensin II on aldosterone secretion

The major physiological stimulator of aldosterone biosynthesis and secretion in man is angiotensin II which acts as a trophic hormone on the zona glomerulosa of the adrenal cortex (Mulrow, 1966; Davis, 1967).

Aldosterone is a potent mineralocorticoid and specifically affects the transport of electrolytes across epithelial surfaces. In the distal tubule of the nephron it induces sodium retention which results in a retention of water and an increase in the plasma volume and renal arterial pressure (Mulrow, 1969). This in turn supresses the secretion of renin which leads to a fall in the concentration of angiotensin II and thereby a reduced secretion of aldosterone by the adrenal. This delicate mechanism provides a negative feedback control of the renin-angiotensin-aldosterone system. The precise locus at which angiotensin II stimulates the secretion of aldosterone is not completely understood. However, it seems likely that it stimulates some step in the steroid biosynthesis between cholesterol and progesterone (Ganong and Van Brunt, 1968; Davis, 1971). The inter-relationships in the renin-angiotensin system can be summarized diagramatically as follows:



Plasma renin level and hypertension

Several diseases in the human are associated with hypertension, and the involvement of renin with these diseases has been comprehensively reviewed by Brown et al (1965a). The hypertension may be associated with a high, normal, or low renin activity as discussed below. It is important to mention here that plasma renin activity is greatly dependent on sodium balance, aldosterone secretion and posture.

Plasma renin levels

The plasma renin level has been found to be elevated in the following hypertensive states: malignant hypertension (Skeggs et al, 1952), nephritis (Boucher et al, 1964), renovascular hypertension (Brown et al, 1964b), renal artery stenosis (Brown et al, 1965a), secondary aldosteronism, hemorrhage (Peart, 1965), sodium losing renal disease, cases of renin

secreting tumors (Brown et al, 1966) and 1-15% of patients with essential hypertension.

The plasma renin level may be normal in 60-70% of the patients with essential hypertension, pheochromocytoma and Cushing's syndrome (Brown et al, 1966), and consistently low in Conn's syndrome (primary aldosteronism) (Conn et al, 1969). More detailed information on this has been provided by Laragh who studied 219 essential hypertensive patients and reported that 27% of these patients had low, 57% normal and 16% high plasma renin activity (Brunner et al, 1972a).

2. Aldosterone

In 1950 Deming and Luetscher reported the presence of a sodium retaining factor in the urine of patients with cardiac failure. The occurrence of this factor was also suggested by studies on electrolytes in sweat and saliva (Conn and Louis, 1950; Berger et al, 1951; Bongiovanni and Eisenmenger, 1951). By 1952, the substance had been isolated from beef adrenals and shown to cause an intense sodium retention in the adrenalectomised rat (Grundy et al, 1952; Simpson et al, 1952). Chemically it proved to be an 18-aldehyde of corticosterone and was called aldosterone (Simpson et al, 1953, 1954).

Aldosterone is a potent mineralocorticoid with about 25-50 times the activity of deoxycorticosterone acetate in the renal retention of sodium by adrenalectomised animals (Simpson et al, 1954). It effects the transport of electrolytes across the epithelial nephron, particularly the distal tubule of the kidney (Vander et al, 1958), the intestinal mucosa and the salivary and sweat glands (Finn and Welt, 1963).

Regulation of aldosterone secretion

Unlike other adrenal steroids, aldosterone is not under the complete control of the pituitary gland. It is now believed that its secretion is regulated by several factors, including the renin-angiotensin system, electrolytes and ACTH; as discussed below.

Renin-angiotensin

The involvement of the renin-angiotensin system in the regulation of aldosterone secretion has been extensively reviewed (Davis, 1963; Blair-West et al, 1963; Ganong and Van Brunt, 1968). Administration of renin or angiotensin II stimulates both aldosterone secretion and the growth of the zona glomerulosa, whereas nephrectomy prevents these effects (Davis et al, 1961b; Mulrow et al, 1962; Mulrow and Ganong, 1964). Infusion of angiotensin II into the jugular vein of man also stimulates aldosterone secretion while direct infusion into the adrenal arterial vein causes a greater and more prolonged effect (Biron et al, 1961; Ganong, 1962a; Ames et al, 1965; Laragh et al, 1964). Ganong and Mulrow (1962b) also found that elevation of endogenous renin due to renal artery constriction leads to an increase in aldosterone secretion.

Electrolytes

There is evidence that the concentration of potassium in the plasma may directly affect aldosterone secretion. Potassium loading increases and depletion decreases its secretion and prevents the stimulation that normally follows sodium depletion (Laragh and Stoerk, 1957; Eilers and Peterson, 1964; Gann et al, 1964). Perfusion studies with isolated dog adrenals have also indicated that potassium acts directly on the adrenal gland (Blair-West et al, 1962; Davis et al, 1964).

It is unlikely that the concentration of serum sodium plays a direct role in aldosterone production, since in man the sodium level does not alter significantly during sodium depletion. However, several other electrolytes have been found to influence aldosterone secretion. For example, magnesium deficiency stimulates aldosterone secretion in the rat. (Ginn et al, 1967), while cesium, rubidium, and ammonium stimulate its in vitro formation by rat adrenal slices (Muller, 1965a,b) and perfused dog adrenal (Bartter et al, 1964).

Adrenocorticotropin

ACTH does not appear to directly control the production of aldosterone since hypophysectomy fails to cause atrophy of the zona glomerulosa of the adrenal gland, unlike the inner zones of the adrenal cortex (Deane, 1962; Laidlaw, 1973). Large doses of ACTH will however stimulate aldosterone secretion initially in the human, but the rate soon returns to the control level with continued administration (Liddle et al, 1956; Forsham et al, 1961; Tucci et al, 1967).

In sodium depleted rats an intact pituitary seems to be essential for normal stimulation and maintenance of aldosterone production, since hypophysectomy impairs aldosterone production in this situation. But the steroidogenic effect of ACTH on aldosterone secretion is not the same as it is for glucocorticoids. It has been suggested that in addition to ACTH, other pituitary factors are necessary for the aldosterone response to sodium depletion (Palmore et al, 1970). In dog, however, a large dose of ACTH alone produces an elevation in aldosterone production (Ganong and Van Brunt, 1968).

Aldosterone and hypertension

Aldosterone does not induce hypertension directly in man, but through secondary alterations in sodium metabolism. Its effect on sodium and water retention increases the extra cellular fluid volume which may then lead to the development of hypertension. It has been suggested that an increase in the

sodium and water content of the renal arterioles decreases their radii, and thereby increases their resistance to flow which produces the hypertension (Finn and Welt, 1963; Conn, 1964a). Hypersecretion of aldosterone has been associated with hypertension in several conditions which may be divided into two groups, those with increased plasma renin activity, and those with low plasma renin activity. The first group includes: secondary aldosteronism, renal artery stenosis, malignant hypertension, renin secreting tumor, and some individuals receiving oral contraceptive pills. The low renin group includes: primary aldosteronism, adrenocortical hyperplasia, and Cushing's syndrome (Bartter et al, 1961; Laidlaw, 1973). In addition to the indirect actions of aldosterone, it may have a direct effect on blood pressure since it has recently been demonstrated that infusion of this hormone into decerebrated rabbits on normal, high and low salt diets, induces an elevation in blood pressure (Burstyn et al, 1971). The mechanism of this action is obscure, but may involve an effect on the kidney or baroreceptor sensitivity.

(a) Primary aldosteronism (Conn's Syndrome)

The term primary aldosteronism implies that the oversecretion of aldosterone arises from within the adrenal cortex, and is typically associated with an aldosterone producing tumor. Primary aldosteronism has also been called Conn's syndrome in recognition of the classical identification of this syndrome (Conn, 1955a,b). The first diagnosis was made on a 34 year old woman who presented with the symptoms of severe weakness, low serum potassium and elevated blood pressure. At surgery a large golden-yellow tumor of the adrenal was observed and excised, and by the tenth post-operative day, all symptoms had disappeared. Conn (1961) has suggested that this patient presents a classical example of hypokalemic primary aldosteronism which can be distinguished by the following diagnostic criteria: hypertension, hypokalemia, normal 17-hydroxy-corticosteroid and 17-ketosteroid excretion, normal renal arteriogram, excessive urinary aldosterone, neutral or alkaline urine of large volume and low

specific gravity, continuous weakness, nocturia and headache. In few cases of primary aldosteronism however, the tumors have been found to produce excessive amounts of corticosterone, aldosterone (Mader and Iseri, 1955; Ayres et al, 1958; Neher, 1958), and 18-hydroxycorticosterone (Abbott et al, 1966).

Patients with primary aldosteronism have been found to have suppressed plasma renin activity (Genest et al, 1964; Conn et al, 1964b) and Conn has suggested that the overproduction of aldosterone together with suppressed renin activity is uniquely diagnostic of primary aldosteronism.

In some cases primary aldosteronism may not be present with all the features given above. Normokalemia has been associated with primary aldosteronism in several reports (Conn et al, 1964b; 1965a; 1966; 1967). However, a recent review states that only 8% of the normokalemic patients actually had primary aldosteronism (Rovner, 1970a). These patients have been suggested to represent early stages of primary aldosteronism before significant alterations in their blood potassium levels have occurred.

A few isolated cases of primary aldosteronism with high aldosterone production but without an adrenal tumor have also been reported, where both the zona glomerulosa and fasciculata were found to be hyperplastic at surgery (Van Buchem et al, 1956; Kretchmer et al, 1959; Moran et al, 1960; Genest et al, 1960b).

On the basis of the observation that plasma renin activity may be suppressed in a significant percentage of patients with essential hypertension, Conn originally predicted that as many as 15-20% of the essential hypertensive population may have undiagnosed adrenal adenomas. He also found that the disease was 2½ times more common in women than in men, and that 70% of the patients were between 30-49 years of age (Conn, 1961). However, a high incidence of adrenal adenomas among the essential hypertensive population has not been confirmed by other workers. In one study, 113 hypertensive cases were

investigated and a mean of 4.4% found to have elevated aldosterone production with increased serum renin levels (Ehrlich, 1968). A further 13.3% had increased serum renin with normal aldosterone, 11.8% had low renin with normal aldosterone, but no cases had primary aldosteronism as defined by Conn. More recently, Rovner (1970b) from Conn's laboratory, reported that 8% of a group of unselected hypertensive patients had primary aldosteronism. By contrast, Kaplan (1970) believes that primary aldosteronism is a rare disease and probably accounts for less than 1% of the essential hypertensive population.

(b) Secondary aldosteronism

Secondary aldosteronism refers to the hypersecretion of aldosterone secondary to stimulation from sources outside the adrenal gland. In 1960 Gross postulated that renin exerts a control function on the secretion of aldosterone and maintains electrolyte balance. Later, increased aldosterone excretion (Genest et al, 1960a) and secretion (Laragh et al, 1960a,b) were observed in hypertensive subjects, and the infusion of angiotensin II into a normal man was shown to also increase the secretion and excretion of aldosterone. These findings provided a link between the kidney, hypertension and aldosterone. Three different factors which may be involved in the stimulation of aldosterone secretion in secondary aldosteronism, are angiotensin II, ACTH and potassium. These effects are considered below.

Angiotensin II

It is now well established that the renin-angiotensin system is a major controlling factor in aldosterone secretion (Davis, 1963; Mulrow and Ganong, 1964; Gross et al, 1965; Ganong et al, 1966; Peart, 1970). Several factors that affect the level of angiotensin II will also affect the secretion of aldosterone. The most common stimulator of angiotensin II formation is decreased pressure in the renal arterioles, which may be caused by a decrease in the effective arterial blood volume or, by obstruction of the blood flow into the kidney. A decrease in extracellular fluid volume also leads to a decrease in the effective arterial blood volume which, in turn, elevates the renin

secretion. The increased plasma renin concentration leads to an increased level of angiotensin II, which stimulates aldosterone secretion by the adrenal cortex. Such a situation occurs in renal artery stenosis producing renovascular hypertension (Kaplan, 1969). It may be important to mention here that most renovascular disease does not produce hypertension (Eyler et al, 1962), since most arterial stenosis do not produce enough obstruction of the blood flow to increase the release of renin.

With hypovolemia, which occurs following the acute loss of fluid associated with diuretics, hemorrhage or gastrointestinal disturbances, there is a progressive wastage of salt and activation of the renin-angiotensin-aldosterone system.

A similar situation is found in cirrhosis of the liver with ascites. A progressive decrease in the effective blood volume leads to very high levels of renin, angiotensin and aldosterone, and to sodium retention and potassium excretion.

An unusual situation where excess angiotensin is produced is due to an inherent lack of sensitivity to endogenous angiotensin through the failure of the blood vessels to respond in a normal manner. This results in an increased angiotensin II production which stimulates aldosterone secretion and is accompanied by hypokalemia and alkalosis, but hypertension is absent. (Bartter, 1962; Martin and White, 1972).

Increased plasma levels of aldosterone are found during pregnancy and in women taking oral contraceptives, but complications do not usually occur since the majority of the aldosterone is bound to protein (Layne et al, 1962; Kean et al, 1969; Horne et al, 1970). There are also isolated reports of renal tumors producing excessive amounts of renin, which in turn elevates the angiotensin II level and produces a secondary aldosteronism (Robertson et al, 1967; Hoshino and Seida, 1968).

Adrenocorticotropin (ACTH)

Whereas ACTH is known to be a major hormone for adrenocorticoids in man, its role in the stimulation of aldosterone synthesis is of less importance. A stimulatory effect of ACTH on aldosterone secretion has however been demonstrated in the rat (Singer and Stack-Dunne, 1955), sheep (Blair-West et al, 1962), ox (Kaplan and Bartter, 1962), dog (Mulrow and Ganong, 1961) and also in man with large doses (Forsham, 1961). In man it appears most likely that ACTH functions mainly in a supporting role in the secretion of aldosterone (Davis, 1961a).

In certain situations, secondary aldosteronism may be associated with elevated ACTH secretion due to lack of cortisol synthesis. Cortisol synthesis is blocked in congenital adrenal hyperplasia due to inborn enzymatic defects. Hexe the negative feed back mechanism between the pituitary and adrenal cortex is not operative, which leads to excessive production of ACTH which in turn causes a hypersecretion of aldosterone (New & Peterson, 1967; Sutherland et al, 1966). Recently it has been suggested from indirect evidence that there are hormones other than ACTH which may cause a hyperproduction of aldosterone in this situation (New et al, 1973).

In situations of surgical stress or acute hemorrhage, it has also been suggested that ACTH plays a role in the stimulation of aldosterone synthesis (Ganong et al, 1966).

Potassium

Serum potassium concentrations appear to have a marked influence on aldosterone secretion. It has been demonstrated both in vitro and by perfusion studies with the adrenal, that potassium acts directly on the adrenal gland (Blair-West et al, 1962; Davis et al, 1964). Elevations in serum potassium increase and depletions decrease aldosterone secretion. Potassium depletion also prevents the rise in aldosterone due to sodium depletion (Laragh and Stoerk, 1957; Davis et al, 1963; Gann et al, 1964), and patients

given exogenous potassium or suffering from a disease associated with hyperkalemia, exhibit a form of secondary aldosteronism.

3. Glucocorticoids

(a) Cushing's Syndrome

The possibility of a relationship between the pathogenesis of hypertension and the adrenal cortex, was suggested by the association of high blood pressure with Cushing's syndrome.

This disease was first described by Cushing in 1932, and was attributed at that time to pituitary hyperactivity due to a basophilic tumor of the pituitary. Later evidence indicated that this disease could be associated with increased cortisol secretion in the absence of pituitary tumor and with bilateral adrenal hyperplasia (Soffer et al, 1957). Cushing's syndrome may be associated with an adenoma or carcinoma of the adrenal cortex (Soffer et al, 1961), excess ACTH production from a pituitary tumor (Salassa et al, 1959), or overactivity of the basophil cells of the pituitary gland (Marks, 1959; Simkin et al, 1962). In each case there is an excessive production of cortisol associated with hypertension (Scholz et al, 1957; Peart, 1967). Aldosterone secretion is usually low (Christy and Laragh, 1961; Peart, 1967), and removal of the source of excessive cortisol relieves the hypertension in most cases (Raker et al, 1964).

The mechanism, by which cortisol or corticosterone increase the blood pressure is not clear since they are not potent pressor agents. The effect has been attributed to their weak mineralocorticoid activity, which is observed when they are administered in high doses (Christy and Laragh, 1961).

More recently, elevated secretion of deoxycorticosterone has been observed in some patients with Cushing's syndrome (Crane and Harris, 1966).

(b) Congenital adrenal hyperplasia

Congenital forms of adrenal hyperplasia are inherited diseases characterised by defects in the biosynthesis of corticosteroids by the adrenal cortex, and are usually identified by clinical signs and symptoms of excessive sex hormone production. In addition, some types are associated with electrolyte imbalance, hypertension and hypoglycemia.

Congenital adrenal hyperplasia has been identified in association with six different enzyme defects (Bondy, 1969), two of which are associated with hypertension; namely, 17α-hydroxylase and 11β-hydroxylase deficiencies.

17α-hydroxylase deficiency results in an inability of the adrenal cortex to produce cortisol, so that excessive amounts of corticosterone and deoxycorticosterone are produced (Biglieri et al, 1966; Goldsmith et al, 1967). Since these latter steroids have no effect on the hypothalamic control of ACTH release, and the negative feedback of cortisol is absent, the production of ACTH increases and leads to a buildup of cortisol precusors. Aldosterone is usually not detectable because of the salt retaining effect of the deoxycorticosterone which suppresses its formation through suppression of renin secretion. The mineralocorticoid action of deoxycorticosterone is also responsible for the hypertension associated with this enzyme defect.

11β-hydroxylase deficiency again results in an inability to produce cortisol and excessive formation of deoxycorticosterone and androgens (Eberlein and Bongiovanni, 1955; Camecho et al, 1966; Gabrilove et al, 1965). The hypersecretion of the deoxycorticosterone is responsible for the hypertension. The associated excessive production of androgens results in masculinisation.

4. Pheochromocytoma

The adrenal medulla is a modified form of the autonomic sympathetic nervous system, and has a metabolic link with the sympathetic nerve endings, which store and release the catecholamines, epinephrine and norepinephrine.

This organ is not often associated with disease; however tumors of the chromaffin cells of the medulla have been shown to secrete elevated levels of catecholamines, a condition that has been classified as pheochromocytoma.

Pheochromocytoma is characterised by excessive production, storage and release of catecholamines, hypertension, increased heart beat, sweating and hyperglycemia. The arterial pressure may rise as high as 300/180 mm Hg (Page and Copeland, 1968; Crout, 1966).

The main secretory product of the medullary tumor in pheochromocytoma is norepinephrine; however, on rare occasions, tumors have been observed to secrete epinephrine, hydroxytyramine (Engel and von Euler, 1950) and dopamine (Robinson et al, 1964). Pheochromocytoma may also occur without an elevation in blood pressure (Leather et al, 1962).

The diagnosis of pheochromocytoma is usually based on the urinary excretion of vanillyl mandelic acid (VMA), a metabolite of epinephrine and norepinephrine, and in addition, on the levels of free catecholamines in urine or blood. Estimation of VMA reportedly detects 90-95% of all cases (Gitlow et al, 1961). The cardiovascular manifestations of pheochromocytoma are primarily due to the excessive production of catecholamines; however, there are other factors which can modify the degree of hypertension caused by the circulating catecholamines. Patients with pheochromocytoma have a greater response to norepinephrine and its vasoconstriction effect produces a greater change in their blood volumes than normal, resulting in an increase in blood pressure (Brunjes et al, 1960; Engleman and Sjoerdsma, 1964). The actual mechanism by which the high level of norepinephrine is maintained in pheochromocytoma is not clear. It has been shown that drugs like tyramine and reserpine bind at the nerve endings, causing a release of norepinephrine into the circulation. The drug, α-methyl dopa is converted into α-methyl norepinephrine and substitutes for norepinephrine at its storage site, which causes an elevation of the level of circulating norepinephrine. These findings indicate that

release of norepinephrine in pheochromocytoma may be induced by the metabolism of other compounds present in the circulatory system (Kopin and Gordon, 1962; Trendelenburg, 1963; Muscholl and Maitre, 1963).

5. Steroids, electrolytes and experimental hypertension

The concept of the adrenal being an important participant in the genesis of hypertension probably dates from Selye's hypothesis (Selye, 1946) that an abnormal reaction of the adrenal cortex to stresses in the environment could lead to hypertensive disease. While evidence for a relationship between stress and the development of hypertension is circumstantial, the involvement of adrenocorticoids, particularly cortisol, aldosterone and deoxycorticosterone in certain types of hypertensive disease has been well documented, and several endocrinopathies have been described where overproduction of endogenous steroids has been accompanied by hypertension; such as cortisol in Cushing's disease, aldosterone in primary aldosteronism, and androgens and deoxycorticosterone in congenital adrenal hyperplasia. The administration of steroids to humans in excessive amounts has also been shown to produce hypertension among other effects (Biglieri et al, 1968, 1970; Perera et al, 1944, 1947). However, studies on the development of experimental hypertension have been exclusively confined to animals for obvious reasons.

The adrenal cortex has received particular attention in the experimental development of hypertension. When regeneration of enucleated rat adrenals was delayed or prevented, the hypertension that normally accompanied this process was also delayed or prevented (Chappel et al, 1958; Skelton et al, 1956); and rats treated with androgens failed to become hypertensive if they had been previously adrenalectomised (Salgado and Selye, 1954).

The literature on experimental hypertension is particularly extensive, but basically falls into the following five categories which will be discussed separately.

(i) Adrenal regeneration hypertension

(ii) Salt and deoxycorticosterone induced hypertension

(iii) Androgen induced hypertension

(iv) Spontaneous hypertension

(v) Renal artery constriction hypertension

(i) Adrenal regeneration hypertension

The development of hypertension in rats bearing one kidney and one regenerating adrenal was observed to require the addition of 1% salt to their drinking water (Skelton, 1955). Regeneration of the adrenal occurred after it had been enucleated by slitting the capsule and gently extruding the medulla and most of the cortex. The hypertension developed in two weeks and was severe by 5-6 weeks. Animals fed a normal diet without added salt developed normal regenerated adrenals whereas those on salt diet had regenerated adrenals with very thin zona glomerulosa (Skelton, 1968). In both cases the adrenal medulla was absent.

The exact mechanism by which the regenerating adrenal initiates hypertension is not known. Hypophysectomy and administration of exogenous corticosterone blocked the development of hypertension (Skelton, 1956, 1959). Endogenous aldosterone or corticosterone could not be implicated in the adrenal regeneration hypertension, since secretion of these compounds was reduced (Brogie and Pellegrino, 1959). However, Skelton (1959) suggested that an abnormal pattern of steroids may be produced by the regenerating adrenal and that these compounds may have the mineralocorticoid activity that is associated with the developing hypertension. Subsequent studies have indicated

that the regenerating adrenal is deficient in both 11β-hydroxylase and 17-hydroxylase enzyme systems which leads to the excessive formation of deoxycorticosterone (Brownie & Skelton, 1965, 1968), and 18-hydroxydeoxycorticosterone (Birmingham et al, 1965, 1968; Rapp, 1970; Melby et al, 1972a). It is likely that these compounds are involved in the development of the hypertension.

(ii) Salt and deoxycorticosterone induced hypertension

Salt and hypertension

The induction of hypertension in rats by adding salt to their drinking water or food was first observed by Sapirstein et al (1950) and later confirmed by others (Meneely et al, 1954; Meneely and Dahl, 1961; Dahl and Schackow, 1964; Hall and Hall, 1969). Rats given a 1% salt drinking solution started to develop hypertension by the third week, and by the eighth week all animals had become hypertensive. Dahl and Schackow (1964) observed that if salt feeding was started early in the life of an animal, the severity of hypertension was greater. Development of hypertension was faster in males than in females, and the longer the period of salt intake, the greater the severity of the hypertension. Histological changes were also found in the heart and kidney, and the renal tubules were dilated.

Deoxycorticosterone and hypertension

Selye (1951) demonstrated that the administration of deoxycorticosterone acetate alone to rats with one kidney removed produced hypertension. Implantation of deoxycorticosterone acetate pellets into rats on normal diets also produced an elevation in blood pressure (Eades et al, 1965; Hall and Hall, 1969).

Salt-deoxycorticosterone and hypertension

With the simultaneous administration of salt and deoxycorticosterone acetate, hypertension developed much faster than with salt or deoxycorticosterone alone. The hypertension usually starts to develop by the 18th day, and by the 4th week all the animals are hypertensive (Knowlton et al, 1952; Ledingham, 1954; Gross and Lichten, 1958; Hall and Hall, 1969).

The mechanism by which salt and deoxycorticosterone induce hypertension is not known. It is speculated that sodium and water retention increase the extracellular fluid and that changes in renal function are involved. Champlain (1969) reported that high salt intake reduced the norepinephrine storage capacity at the nerve endings and results in increased amounts of physiologically active norepinephrine being released into the circulation, which contributes to the development of the hypertension. Recently Belin and Ziaka (1972) reported that high salt and deoxycorticosterone administration resulted in a thickening of the arteriolar walls which led to an increased peripheral resistance to the blood flow and was associated with hypertension.

Salt susceptible(S) and resistant(R) rats

During experiments on salt induced hypertension, Dahl et al (1962a,b) observed that some rats developed a permanent hypertension in two weeks and others in 1-2 months, while some never became hypertensive. This raised the possibility that these variations in sensitivity to salt were controlled by genetic factor(s) and that two strains of rat could be developed by selective inbreeding. The most sensitive(S) and most resistant(R) animals with the highest and lowest blood pressures respectively from the first generation were therefore bred. By the third generation the response of the two strains to salt was significantly different, and was most marked by the fifth generation. The R rats when fed a high salt diet did not develop hypertension, while the S rats on a low salt diet easily developed hypertension. When salt and deoxycorticosterone acetate were administered simultaneously to the S and R rats, it was observed that hypertension started to develop by the third week in the S group, and by the eighth week was so severe that all the animals died. The blood pressure of the resistant rats started to rise by the fourth week and was only moderately increased by the twelfth week with all the animals alive(Dahl et al, 1963).

Rapp and Dahl (1971) and Dahl et al (1972) have found markedly high 18-hydroxydeoxycorticosterone but reduced corticosterone secretion by the S rats compared to the R rats, and have suggested that the S rats have an increased 18-hydroxylase activity and a decreased 11-hydroxylase activity. This difference was attributed to one or more genetic control factor(s).

Iwai et al (1970, 1972) reported that a low salt diet increased the sensitivity of the S rats to angiotensin and decreased their sensitivity to renin in relation to blood pressure. In the same group of animals, high salt diet increased the response to renin and angiotensin, and bilateral nephrectomy caused an increased response to renin but a decreased response to angiotensin. The R rats did not exhibit any of these effects. In view of these findings they proposed that a renin inhibitor (a hypertensionogenic factor) may be involved in the development of hypertension by increasing the sensitivity of the S rats to angiotensin.

(iii) Androgen induced hypertension

In 1953 Skelton reported that administration of methyl androstenediol to uninephrectomised rats receiving a 1% saline drinking solution produced hypertension. Subsequently it was shown that both methyl testosterone and testosterone were effective in inducing hypertension in rats (Skelton et al, 1969, Colby et al, 1970). However, Salgado and Selye (1954) found that the hypertension failed to develop when methyl androstenediol was given to adrenalectomised rats.

The androgen induced hypertension is also associated with cardiac and renal hypertrophy, adrenal and pituitary atrophy, and vascular lesions. The kidneys of testosterone treated animals contained tubular casts and showed degeneration in the tubular epithelium (Skelton, 1969; Colby, 1970).

Incubation of progesterone with adrenal homogenates from rats treated with methyl androstenediol gave deoxycorticosterone as the major product (Brownie and Skelton, 1968). Further in vitro studies with adrenal homogenates from androgen treated rats and electron microscopic studies have revealed a low level of cytochrome P450 and a deficiency in both 11β- and 18-hydroxylase activities (Skelton, 1969; Colby, 1970). These findings suggest that the hypertension induced in these animals by androgen administration results from an inadequacy of certain adrenal cortical enzymes resulting in an excessive secretion of deoxycorticosterone, which together with the ingestion of salt, leads to the development of the hypertensive state.

(iv) Spontaneous hypertension

Essential hypertension in the human is not associated with any obvious defect which may be said to initiate the high blood pressure. A model animal strain has been developed for the study of essential hypertension where the elevation in blood pressure also develops spontaneously without apparent cause.

Hypertensive Wistar rats have been selectively bred to the twenty-first generation to obtain a genetic strain of spontaneously hypertensive rat (SHR) (Okomoto & Aoki, 1963). Hypertensive cardiovascular disease commonly occurs at an advanced stage of the hypertension and these animals also exhibit low plasma renin activity, adrenal hyperplasia and hypokalemia (Sokabe, 1966).

Louis et al (1969) studied the effect of administration of salt on the blood pressure of the SHR and concluded that these rats were insensitive to high salt intake. The plasma potassium levels of the SHR were found to be 10% lower than the controls, and sodium depletion for 4 weeks did not reduce the blood pressure (Baer et al, 1972). Administration of exogenous corticosterone for 8 weeks delayed the development of the hypertension but did not prevent it.

Total adrenalectomy also failed to prevent the development of the hypertension. These findings suggest that the sodium balance and adrenal cortex were not of primary importance in the development of hypertension in the SHR. In addition, the levels of 18-hydroxydeoxycorticosterone, deoxycorticosterone and aldosterone in the adrenal venous blood of the SHR were not altered (Melby et al, 1972b).

(v) Renal artery constriction hypertension

Attempts have been made to reproduce hypertension in animals by damaging the kidney or by obstructing its blood supply. Goldblatt (1934) in the first classical experiment showed that constriction of a renal artery in the dog leaving the other kidney intact, produced hypertension. A silver clip applied to the renal artery caused the blood pressure to rise within 24-72 hours and gradually reach a plateau by 1-2 months. A similar observation was made on rabbits (Pickering and Prinzmetal, 1938), and rats (Wilson and Byrom, 1939). In the dog, the hypertension persisted for several years after the renal artery was constricted. However, if both renal arteries are constricted, or one is constricted and the other removed, a severe permanent hypertension is produced.

The hypertension that is caused by renal artery constriction can be relieved by removing the clip and restoring the blood flow or by removing the affected kidney (Goldblatt, 1938; Pickering, 1945). These early observations indicated that hypertension induced by renal artery constriction was due to an increased peripheral resistance. McCubbin et al (1956) showed that the carotid baroreceptors become reset at a new level of arterial pressure in this situation.

Electrolyte estimations in renal artery constriction hypertension revealed that the levels of sodium and potassium were high in the aorta (Tobian, 1956; Phelan and Wong, 1968), arteries (Koletsky et al, 1959) and arterioles (Tobian et al, 1961, 1967).

Studies on the involvement of the renin-angiotensin system, suggested that it was not directly responsible for the elevation in blood pressure, since no changes in plasma renin activity occurred in the dog (Fasciolo et al, 1964), sheep (Blair-West et al, 1968), rat (Gross et al, 1964b), or rabbit (Peart et al, 1961).

Ayers et al (1969) studied the effect of vasodepressor and vasodilator drugs on plasma renin activity in renal artery constriction hypertension. They found that the plasma renin activity was markedly increased with vasodepressor drugs, such as reserpine. In control animals, the plasma renin activity was initially elevated but decreased with increasing blood pressure. It was suggested that renal constriction causes vasodilation which releases the renin, and is followed by vasoconstriction which inhibits further release.

6. Estrogen, progestogens and hypertension

Hypertension, which may occur as a complication of pregnancy is usually associated with sodium and water retention. Although the plasma level and urinary excretion of aldosterone are increased (Jones et al, 1959; Vande Wiele et al, 1960; Layne et al, 1962; Sims et al, 1964), the level of physiologically active aldosterone is not significantly altered because the majority is bound to protein. This may be the result of the stimulation of protein synthesis by estrogens (Layne et al, 1962; Kean et al, 1969; and Horne et al, 1970).

Pregnancy is also associated with consistantly marked increased production of renin substrate, but with inconsistant increases in plasma renin activity or concentration (Newton et al, 1968; Skinner et al, 1969; Wienberger et al, 1969; Nasjletti et al, 1970). In this respect the source of renin is of some interest, since renin-like activity has been found in human amniotic fluid (Brown, 1964a) and the placenta and uterus of the rabbit

(Gross, 1964a) and the pig (Gould, 1964).

It is not clear whether the high levels of progesterone that are produced during pregnancy have any definite relationship with the increased biosynthesis and secretion of aldosterone in this situation (Laragh et al, 1964). However, it is known that progesterone and aldosterone have opposing action on the renal excretion of sodium. Progesterone reduces the tubular reabsorption of sodium and stimulates its excretion by competing with aldosterone for binding sites in the kidney (Landau et al, 1955; Landau and Lugibihl, 1958, 1961). Recently this theory has been supported by Laidlaw (1973) in relation to the effect of aldactone on aldosterone production.

Hypertension has also been found to occur in a small proportion of the population taking oral contraceptives. These preparations contain various estrogen and progesterone analogs, which probably are responsible for the hypertension, as well as for other changes that are normally associated with pregnancy: such as elevated plasma levels of renin-substrate and renin. It has been shown in humans that progesterone alone has very little effect on renin-substrate concentration (Newton et al, 1968), but estrogen markedly increases it. Laragh (1972) has proposed that at extremely high levels of renin-substrate there may be a failure or inadequate suppression of the secretion of renin. Some patients taking estrogen contraceptives also have markedly elevated plasma renin activities, and a direct stimulatory effect of estrogen on the secretion of renin has been proposed. It seems likely, therefore, that the estrogen component of oral contraceptives may be the potent stimulator of the renin-substrate levels (Weir et al, 1970).

Estrogen, and estrogen-progesterone combinations, have been shown to increase cortisol binding protein, aldosterone binding protein and α_2-macroglobulin (Layne et al, 1962; Kean et al, 1969; Horne et al, 1970). Since angiotensinogen (renin-substrate) is in the α_2-globulin fraction, it seems possible that the increased concentration of this substrate is associated with the increased hepatic protein synthesis that is stimulated by oral contraceptives.

7. Steroid metabolism and Essential Hypertension:

Background to thesis

There is considerable evidence to support the involvement of the adrenal cortex in the etiology of hypertension. Although the association of hypertension with aldosterone and cortisol in primary aldosteronism and Cushing's syndrome respectively, is fairly well understood, as discussed previously, the relationship between alterations in the peripheral metabolism of steroids and benign essential hypertension remains obscure.

Several reports have indicated that the urinary excretion rate of a variety of steroids may be altered in patients with essential, malignant and renal hypertension. Thus, the urinary excretion of pregnanediol (Vermeulen and Van der Straeten, 1963), pregnanetriol and Δ^5-pregnentriol (Vermeulen and Van der Straeten, 1963; Nowaczynski et al, 1960, 1964), and dehydroepiandrosterone sulfate (Nowaczynski et al, 1968; Dey et al, 1972) is significantly reduced in essential hypertensives as compared to normotensive subjects. By contrast, the secretion rate of DHEA and DHEA-sulfate is 5 and 6 times greater respectively in hypertensive patients (Shao et al, 1970), and the level of progesterone in the plasma is more than doubled (Sasaki et al, 1972, Genest et al, 1972).

Consideration of these findings has led Nowaczynski et al (1968) to conclude that the differences observed between the groups of normal individuals and hypertensives are more pronounced after 17α-hydroxylation than before. In the following diagram which is modified from that of Nowaczynski et al (1968) and incorporates several other worker's findings, gives the biosynthetic and metabolic pathways of several adrenal steroids and the differences found in the hypertensive patients. Other workers have also suggested that there may be a 17α-hydroxylation deficiency in the adrenal cortex of hypertensive patients from consideration of the fall in the hydrocortisone-corticosterone ratio which was observed with increasing severity of hypertension (Cooper et al, 1958).

Biglieri et al (1966) have described a patient with 17α-hydroxylation deficiency who produced excess corticosterone and deoxycorticosterone, and had hypertension and hypokalemic alkalosis.

The excretion of DHEA-sulfate in the urine of essential hypertensives has been found to be highly significantly reduced from a mean of 3.32mg/24hr in the normal subjects, to 0.16mg/24hr in the hypertensives (Nowaczynski et al, 1968). Both DHEA and DHEA-sulfate are secreted primarily by the adrenal (Baulieu et al, 1965; Wieland, 1965; Weinheimer et al, 1966; Gandy and Peterson, 1968; Nieschlag et al, 1973) and Shao et al (1970) have given the secretion rate of DHEA and DHEA-sulfate in normal subjects as 9.8 and 10.7mg/day respectively. Although DHEA has been isolated from the testis in very limited quantity (Neher and Wettstein, 1960; Axelrod, 1965; Gandy and Peterson, 1968), there is agreement that the normal testis does not secrete DHEA-sulfate (DeMoor and Heyns, 1966; Laatikainen et al, 1971; Nieschlag et al, 1973). Ovarian secretion of DHEA has been suggested (Aakvaag and Fylling, 1968) but several studies have refuted this claim (Kalliala et al, 1970; Nieschlag et al, 1973). DHEA-sulfate may undergo direct metabolism without cleavage of the conjugate or indirect metabolism following removal of the sulfate. In the latter case the two major metabolites are androsterone and etiocholanolone. In addition, DHEA occupies an unique position as a precursor of other androgens, since it can undergo transformation into Δ^4-androstenedione and then to testosterone in the testis. Both of these two steroids are further metabolized to androsterone and etiocholanolone and their "diols" (Dorfman and Unger, 1965).

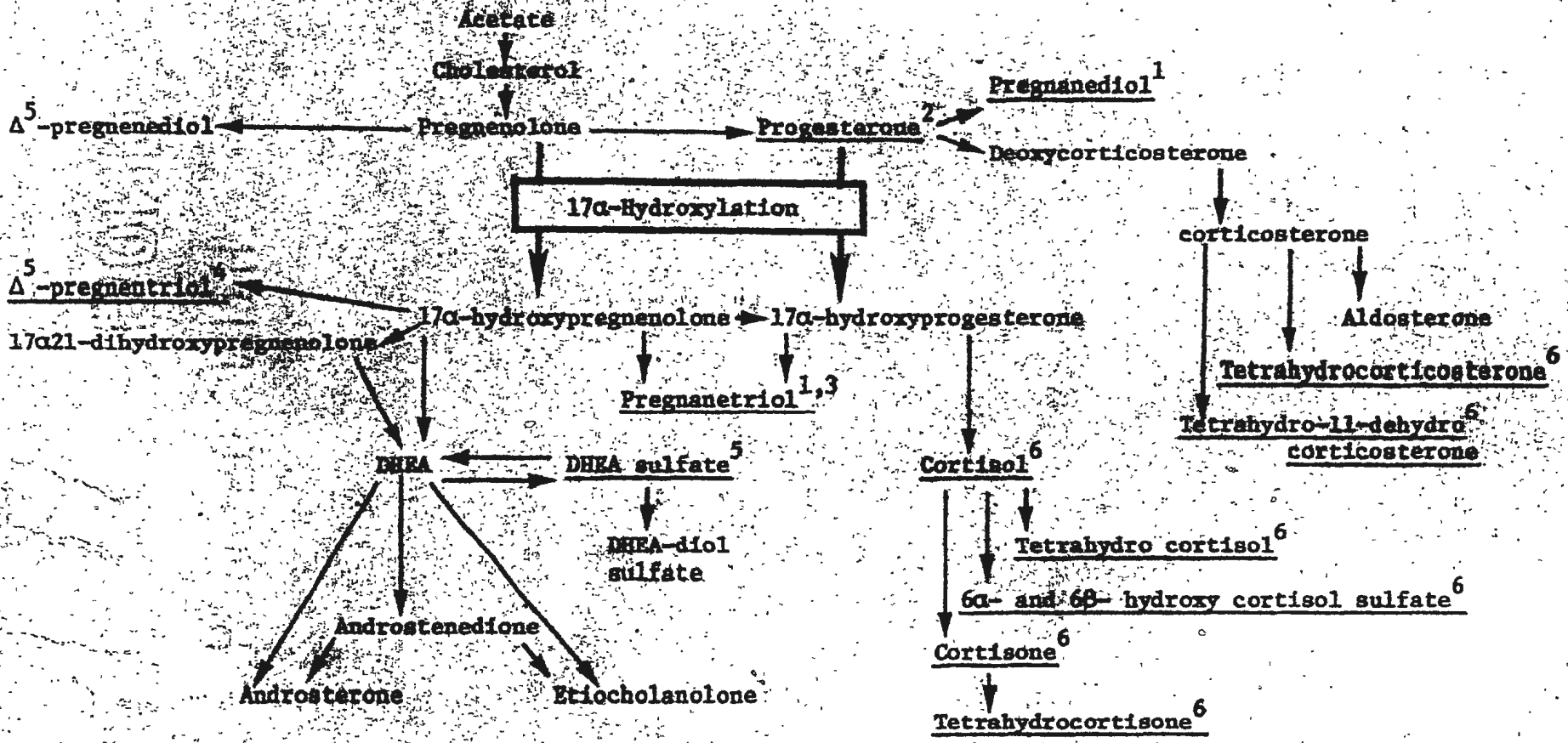
An increased excretion of DHEA has also been found to be associated with oliguria and hypertension (Bassoe et al, 1965), and Sonka et al (1965) have shown that the total excretion of DHEA (glucuronide and sulfate) is increased in uncomplicated obesity, in contrast to obesity complicated by diabetes and hypertension where it was found to be sharply decreased. However,

neither DHEA or DHEA-sulfate showed any activity when administered to humans by continuous intravenous infusion (Lipsett et al, 1965).

The metabolism of corticosteroids in essential hypertension has been most extensively studied by Kornel and his associates who fractionated the metabolites into several groups (Kornel & Motohashi, 1965; Kornel and Takeda, 1967; Kornel et al, 1969). The following statistically significant differences were found between the normal and essential hypertensive groups: (1) in the free steroid fraction, excretion of cortisol and cortisone was higher in hypertensives, (2) in the glucuronide conjugated fraction, excretion of tetrahydro cortisol, tetrahydro cortisone, tetrahydro corticosterone and tetrahydro-11-dehydro corticosterone was lower in the hypertensives, (3) in the sulfate fraction, excretion of the most polar steroids, 6α- and 6β-hydroxy cortisol, was higher in the hypertensives. Consideration of these findings led Kornel to propose that patients with essential hypertension, exhibit a defect in the steroid ring-A reducing enzyme systems (Kornel et al, 1969).

In this context it is of interest to note that Silah et al (1970a,b) have recently demonstrated an increased reduction of the ring-A system of both cortisol and corticosterone when livers from hypertensive rats were incubated in vitro. It has furthermore been postulated that high blood pressure, or one or more causative factors, may in some way affect the enzymatic activity involved in the metabolic transformation of corticosteroids by the liver.

An outline of steroid biosynthesis and metabolism involved in essential hypertension*



*Steroids altered in essential hypertension are underlined.

1. Vermeulen and Van der Straeten, 1963
2. Sasaki et al, 1970
3. Nowaczynski et al, 1960
4. Nowaczynski et al, 1964
5. Nowaczynski et al, 1968
6. Kornel et al, 1969

Objectives of thesis

In view of reports that the excretion of DHEA-sulfate in the urine of essential hypertensive patients was markedly reduced (Nowaczynski et al, 1968), whereas the secretion rate of this compound was considerably elevated (Shao et al, 1970), it was of interest to determine whether this was the result of further metabolism. Conceivably these metabolites might have some role in the etiology of the hypertension. The excretion of the major metabolites of DHEA, namely: androsterone and etiocholanolone, are therefore compared in the urine of hypertensive and normal subjects, together with the intermediate compound Δ^4-androstenedione. In addition the spectra of metabolites are examined by gas chromatography in order to determine whether different metabolites are excreted by the hypertensive and normal groups. The estimation of androsterone and etiocholanolone also provides information on the relative 5α- and 5β-reductases that are involved in the metabolism of the $C_{19}O_2$-17-ketosteroids. The influence of the 11-oxygen function on the relative 5α- and 5β-reductase activities is examined by estimating four major $C_{19}O_3$-17-ketosteroids, namely 11β-hydroxy and 11-keto androsterone and etiocholanolone. The proportions of the 11-oxygenated metabolites are also compared in order to determine the relative 11-oxygenation activities in the two groups.

The final section of the thesis compares the influence of the C_{17}-corticoid side chain on the 5α- and 5β-reductase enzyme activities in the normal and hypertensive groups. Conversion of these compounds to 17-ketogenic steroids allows a further measure of the 11-oxygenation index in this class of compounds.

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1. MATERIALS

A. Source of Materials

(a) Solvents

Ethylene chloride, methylene chloride, n-propanol, benzene, cyclohexane, iso-octane, ethylene glycol, t-butanol, pyridine, ethyl acetate, toluene, dioxane, diethyl ether, acetone, tetrahydrofuran, methyl alcohol, absolute and 95% ethyl alcohol were purchased from Fisher Scientific Co., Canada.

Chloroform was purchased from Canadian Laboratory Supplies, Canada; ligroin, B.P. 35-60°C from Eastman Organic Chemicals, U.S.A.; hexane, sequanol grade, from Pierce Chemicals, U.S.A.; and diethylamine from Aldrich Chemicals, U.S.A.

All solvents were either of certified grade or were purified further.

(b) Chemicals

Sodium hydroxide, sodium sulfate, sodium acetate, sodium chloride, sodium borohydride, sodium periodate, potassium hydroxide, m-dinitrobenzene granulated zinc, blue tetrazolium, acetic anhydride, concentrated hydrochloric and sulfuric acids, and glacial acetic acid were purchased from Fisher Scientific Co., Canada.

Picric acid, B.P. was obtained from General Chemicals, U.S.A.; creatinine zinc chloride and tetramethyl ammonium hydroxide, A.R. from Eastman Organic Chemicals, U.S.A.; phenylhydrazine HCl from British Drug Houses, Canada; and hexamethyl disilazane, trimethyl chlorosilane, chloromethyl dimethyl chlorosilane, "DMF-sil-Prep- Kit and siliconized glass-wool from Applied Science, U.S.A.

(c) Chromatography Supplies

Silica gel, GF_{254} and Whatman chromatography papers, number 1,2 and 3mm (57cm length) were purchased from Canadian Laboratory Supplies, Canada; silica gel, grade 950, 60-200 mesh, from Fisher Scientific Co., Canada; silica gel, 100-200 mesh, from Nutritional Biochemicals, U.S.A.; and Amberlite XAD-2 resin from Rohm and Haas, U.S.A.

Gas liquid chromatography (GLC) columns (6'-U-type, glass, 4mm O.D., and 12' - coiled, glass, 4mm O.D.); GLC column packings precoated with 3% XE-60, 3% SE-30, 3% Hi-eff-8BP, 3% QF-1, 1% SE-30 on 100-200 silinized GCQ; septums, "O"-rings and hardwares were purchased from Applied Science, U.S.A. 2% XE-60 on 100-120 Supelcoport was supplied by Supelco, U.S.A. Nitrogen, hydrogen and air were obtained from Canadian Liquid Air, Canada.

(d) Enzymes

β-glucuronidase (Ketodase, beef liver, 5000 Fishman units/ml) was purchased from Warner Chilcott, U.S.A.; and Glusulase (containing 100,000 units β-glucuronidase and 50,000 units sulfatase per ml) from Endo Laboratories, U.S.A.

(e) Steroids

All steroids were purchased from Sigma Chemical Co., U.S.A., except dihydrotestosterone, 5β-androstan-3β-ol-17-one, 5α-androstan-3β-ol-17-one, 5α-androstan-3β,11β-diol-17-one and 5-androsten-3α-ol-17-one, which were purchased from Steraloids, U.S.A.

(f) Isotopically labelled steroids and chemicals

4-^{14}C-D-Aldosterone, 30-50mCi/mM; 4-^{14}C-dehydroepiandrosterone, 50mCi/mM; 1,2-^{3}H-etiocholanolone, 30Ci/mM; 1,2-^{3}H-Δ^{4}-androstene-3,17-dione, 50Ci/mM; 7-^{3}H-dehydroepiandrosterone sulfate, ammonium salt, 5Ci/mM; and 1,2-^{3}H-tetrahydrocortisone, 20Ci/mM were purchased from New England Nuclear, Canada, and ^{3}H-acetic anhydride, 500mCi/mM from the Radiochemical Centre, England.

(g) Scintillation chemicals and supplies

PPO, POPOP and low potassium glass counting vials were purchased from Packard Instruments, U.S.A.

(h) Special glassware

Coleman cells, (round, 25x105mm; 1cm square); glass-stoppered tubes (2.5ml); scintered funnels (medium) and centrifuge tubes, (pyrex, 15ml, conical) were purchased from Canadian Laboratory Supplies, Canada; and silylation tubes, "mini-aktors" (1.5ml), and Hamilton syringes from Applied Science, U.S.A.

B. Purification of solvents and reagents

(a) Solvents

Ethylene chloride and methylene chloride were distilled once rejecting the first and last 10% distillate. Benzene, cyclohexane and isooctane were distilled twice rejecting 10% of the distillate as above.

Methyl alcohol, absolute and 95% ethyl alcohol were refluxed with 6g granulated zinc and 10g KOH per litre of alcohol for 1hr. Each was redistilled twice collecting the middle fraction and discarding the first and last 10% distillate.

Pyridine was refluxed over KOH pellets for 6hr and distilled three times.

Ethyl acetate was washed with 2N-sodium carbonate solution and water, dried over anhydrous sodium sulfate and distilled once (Sarda et al, 1968).

Tetrahydrofuran was refluxed over KOH for 24hr and distilled once. The distillate was percolated through a silica gel column and stored in an amber bottle in a desiccator (France et al, 1965). This solvent was freshly purified each week.

Chloroform was distilled from anhydrous potassium carbonate and 1% purified ethyl alcohol (v/v) was added to prevent the formation of phosgene during storage in an amber bottle.

Small volumes of diethylamine were distilled over KOH pellets before use.

(b) Reagents

M-Dinitrobenzene was recrystallised twice from 95% ethyl alcohol, dried and stored in an amber bottle.

Phenylhydrazine HCl (100g) was dissolved in 500ml hot absolute ethyl alcohol, filtered hot through Whatman No.1 filter paper and crystallised twice. The crystals were washed with cold alcohol, dried, and stored in an amber bottle.

Picric acid was crystallised from methyl alcohol, dried and stored in an amber bottle.

Acetic anhydride was refluxed over calcium carbide for 4hrs and distilled three times.

2. METHODOLOGY

A. Colorimetric assay of creatinine

Procedure

Urinary creatinine was assayed as described by Lambert (1945) and Anker (1954). The urine was diluted 1:15 or 1:25 depending on its volume. Picric acid (2ml, 1.175%) and sodium hydroxide (0.15ml, 2.5N) were added to one ml of the diluted urine, standards and water blank. The reactants were mixed, and after 20min at room temperature, diluted to 15ml with water. Optical densities were read at 520mu with a Coleman Spectrophotometer, Jr. II. Fig. 1 gives a typical calibration curve plotted for standards in the range 0-100ug.

Standards

A stock standard, prepared by dissolving creatinine zinc chloride (400.5mg) in HCl (500ml, 0.1N) was stored in an amber bottle with a few drops of chloroform as preservative. Working standards were prepared by pipetting 1,2,3 and 4ml of the stock standard and diluting each to 20ml with water. One ml of each standard was reacted as above.

Calculation:

$$\text{Creatinine (mg/24hr)} = \frac{\text{Standard (mg)} \times \text{Experiment O.D.} \times \text{Dilution factor} \times \text{Total Urine vol.}}{\text{Standard O.D.}}$$

Urine dilution factor: 15 for 1:15 dilution

25 for 1:25 dilution

B. Colorimetric assay of total 17-ketosteroids

Procedure

Total urinary 17-ketosteroids were assayed by a colorimetric method as described by Ware (1959). Urine (10ml) was hydrolysed in duplicate with conc. HCl (3ml) in a boiling water bath at 95°C for 15 min. After cooling, the hydrolysate was extracted with ethylene chloride (10ml) and centrifuged (5min, 2000 rpm). The ethylene chloride extract was shaken with 10-12 pellets of sodium hydroxide, centrifuged, and the clear extract (5ml) transferred to a 15ml centrifuge tube and evaporated.

Zimmerman color chromogens were developed by adding an alcoholic solution of m-dinitrobenzene (0.2ml, 2%-w/v) and freshly prepared alcoholic KOH (0.2ml, 2.5N) to the dried extracts, standards and blank. The reactants were mixed and allowed to stand for 30 min in the dark. Propanol (6ml, 40%-v/v) and methylene chloride (3ml) were added to each tube, shaken well and centrifuged (5min, 2000 rpm). The optical densities were read at 520mu. Fig. 1 gives a typical calibration curve.

Standards

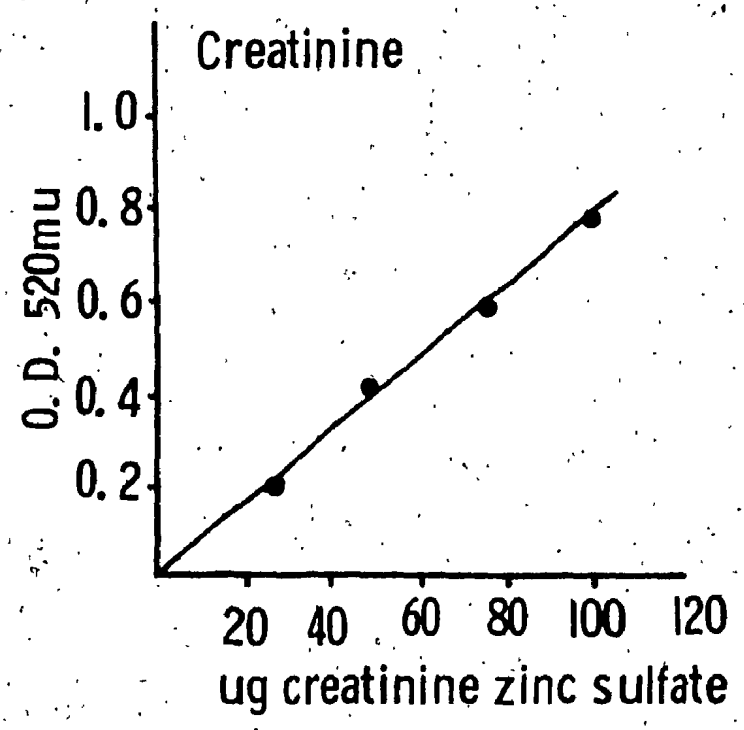
A stock standard was prepared by dissolving DHEA in absolute ethanol (1ug/ul). The working standards for the calibration curve contained 5,10,15,20,30 and 50ug DHEA.

Blank

2% alcoholic solution of m-dinitrobenzene (0.2ml) and alcoholic KOH (0.2ml).

Calculation

$$\text{Total 17-KS (mg/24hr)} = \frac{\text{Standard (mg)} \times \text{Experiment 0.D.} \times \text{Total Urine vol.}}{\text{Standard 0.D.} \times \text{urine volume extracted}}$$



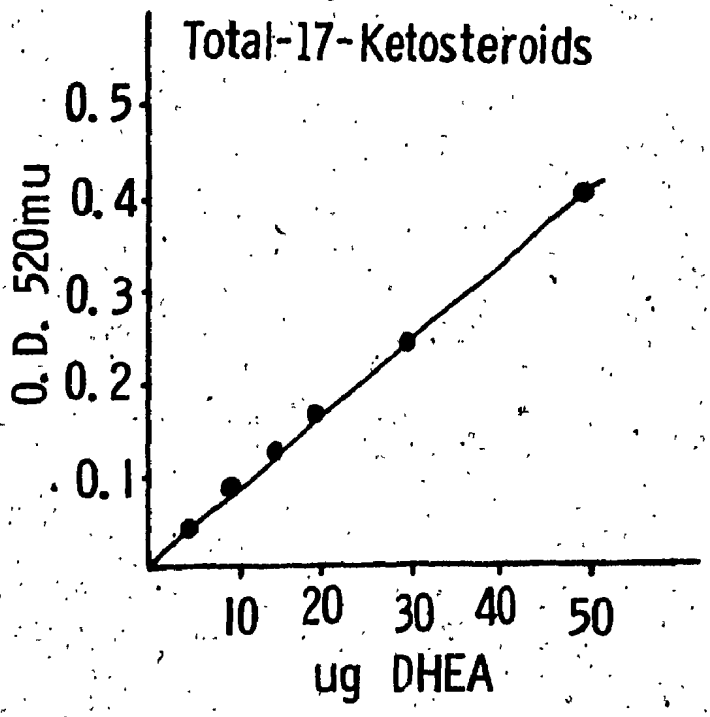


Fig. 1 Calibration curves for Colorimetric assays

C. Colorimetric assay of total 17-hydroxycorticosteroids

Procedure

The total urinary 17-hydroxycorticosteroids were estimated by a colorimetric method taken from Sunderman (1960). Duplicate aliquots of urine (10ml), a water blank (10ml) and a standard cortisol solution (50ug in 10ml) were pipetted into separate measuring cylinders (250ml, glass stoppered). All samples were adjusted to pH5 with acetic acid (5%-v/v) or NaOH (4%-w/v) as required, and incubated with Ketodase (0.2ml) and acetate buffer (2ml, pH5, 1M) with chloroform (0.1ml) added as preservative. After incubation at 37°C for 24hr, the hydrolysate was extracted with chloroform (100ml) and the extract washed with NaOH (10ml, 0.1N) followed by water (10ml).

Duplicate aliquots (40ml) of the clear extracts were transferred to 50ml tubes and reagents added as below:

Blank:	40ml blank extract and 5ml alcoholic sulfuric acid
Blank phenyl:	40ml blank extract and 5ml phenyl hydrazine
Standard blank:	40ml standard extract and 5ml alcoholic sulfuric acid
Standard phenyl:	40ml standard extract and 5ml phenyl hydrazine
Test blank:	40ml test extract and 5ml alcoholic sulfuric acid
Test phenyl:	40ml test extract and 5ml phenyl hydrazine

Alcoholic sulfuric acid: sulfuric acid (64%-v/v) - absolute ethanol (10-5 (v/v))

Phenyl hydrazine: recrystallised phenylhydrazine HCl in absolute ethanol (1mg/ml)

The contents of each tube were mixed and allowed to stand for 20min. An aliquote (2.5ml) was transferred from each tube to a correspondingly labelled

Coleman Cell, and after incubation for 30min at 60°C the optical densities were read at 410mu.

Standards

A stock standard was prepared by dissolving cortisol in absolute ethanol (100ug/ml). A working standard (5ug/ml) was prepared by diluting 5ml of the stock standard to 100ml with water.

Blank: 10ml water

Calculation:

Total 17-hydroxycorticosteroid (mg/24hr)

$$= \frac{(\text{O.D. Test phenyl}) - (\text{O.D. Test blank})}{(\text{O.D. Standard phenyl}) - (\text{O.D. Standard blank}} \times \frac{\text{Total urine Vol}}{10} \times 0.05$$

D. Colorimetric assay of total 17-ketogenic steroids

Procedure

17-ketogenic steroids were measured in the urine by an assay procedure based on the methods of Few (1961), Rutherford and Nelson (1963) and Wilson and Lipsett (1963). Urine (10ml) was taken in duplicate in 50ml tubes and tested for the presence of glucose with "Clinitest" tablets.

If glucose was present, the urine (10ml) was diluted to 25ml with water, ammonium sulfate (12.5gm) added and the mixture extracted (3 x 10ml) with ethanol-ether (1-3). The combined extracts were evaporated and dissolved in urea solution (10ml, 0.2%).

The urine was adjusted to pH7, and reduced with sodium botohydride (1ml, 10% in 0.1N NaOH). After 1hr at room temperature, acetic acid (0.5ml, 25%-v/v) was added and the frothing checked by addition of ether. The pH was readjusted to 7 after standing 15min at room temperature, and the reaction mixture oxidised with sodium periodate (4ml, 10%) and NaOH (0.8ml, 4%). After incubation at 37°C for 1hr, NaOH (0.25ml, 40%) was added and incubation continued for a further 15min. The reaction mixture was cooled, extracted with ethylene chloride (10ml) and the extract washed sequentially with

H_2SO_4 (2.5ml, 5%-v/v), water (2.5ml), sodium hydrosulfite (2.5ml, 5% in 2.5N NaOH) and water (2 x 2.5ml). The extract was dried over anhydrous sodium sulfate (1gm), transferred (5ml) to a 15ml centrifuge tube and evaporated. The total 17-ketogenic steroids were quantitated by the Zimmerman Color reaction as described for the total 17-ketosteroids.

Standards and blank: Same as for total 17-KS

Calculation:

$$\text{Total 17-ketogenic steroids (mg/24hr)} = \frac{\text{Standard (mg)} \times \text{Experiment O.D.} \times \text{Total urine vol.}}{\text{Standard O.D.} \times \text{vol. urine extracted}}$$

E. Double isotope derivative assay of urinary aldosterone

A double isotope derivative assay of aldosterone was developed that involved acetylation with ^{3}H-acetic anhydride of low specific activity and a modified combination of the chromatographic procedures given by Kliman and Peterson (1960) and Nowaczynski et al (1967). The methodology has been reported (Dey et al, 1972). In summary an accurate amount of ^{14}C-aldosterone was added to the urine and the extracted steroid was acetylated with ^{3}H-acetic anhydride. The reaction product, aldosterone-18,21-diacetate, was purified in several chromatographic systems until a constant $^3H/^{14}C$ isotopic ratio was obtained. The quantity of aldosterone was calculated from the final ^{3}H-count, the percentage recovery of ^{14}C, and the specific activity of the ^{3}H-acetic anhydride.

(1) Chromatography systems (all proportions are by volume):

System	Running time (overruns)
(i) Thin layer: chloroform-acetone (3-2)	50-60min
(ii) Thin layer: toluene-ethanol (90-10)	50-60min
(iii) Thin layer: toluene-ethanol (85-15)	50-60min

(iv) Paper: ethyleneglycol/isooctane-toluene (7-3) 16hr

(v) Paper: isooctane-t-butanol-water (5-1-5) 6hr

(vi) Paper: Benzene-cyclohexane-methanol-water (6-4-2-1) 6hr

(vii) Paper: isooctane-benzene-methanol-water (6-4-8-2) 6hr

(viii) Paper: petroleum ether-benzene-methanol-water (33-17-40-10) 3hr

Solvent systems (iv), (v) and (vi) were taken from the reports of Nowaczynski et al (1967); systems (ii), (iii) and (vi) from Kliman and Peterson (1960) and system (viii) from Bush (1961).

(2) Chromatography procedures:

Extreme precautions were taken during the handling of all chromatographic systems and chromatograms to avoid radioactive contamination.

Thin layer chromatograpy (TLC):

Glass plates (20 x 20cm) were coated with silica gel GF_{254} (0.25mm thickness) and dried at 110°C for 1hr. The plates were washed by ascending chromatography with ether and methanol and then reactivated at 110°C and stored in a desiccator over "Drierite".

The dried samples were dissolved in a few drops of benzene-methanol (1-1:v/v) and carefully transferred to TLC plates with fire-polished disposable glass capillary pipettes. Three subsequent rinses assured quantitative transfer. Ascending TLC was run at room temperature in paper lined tanks after equilibration with freshly mixed solvents (total volume 100ml). Steroids were removed from TLC plates by scraping the silica gel into medium scintered funnels and then eluted with methanol-methylene chloride (10-90: v/v).

Paper chromatography:

Whatman papers were washed by descending chromatography with methanol for 48hr and dried before use. Papers were impregnated with ethylene glycol methanol (15-35) for system (iv). System (iv) was run on

Whatman #2 paper and all other systems on Whatman #1 paper. The paper chromatograms were developed by descending chromatography at 28-32°C and the steroids located under u.v. The appropriate zones were cut out, attached to a hypodermic syringe and eluted with methanol (10ml).

(3) Radioactivity counting and scanning procedure:

Radioactivity was located on paper and thin layer plates with a Packard Radiochromatogram scanner (model 7210).

^{3}H and ^{14}C were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer (model 3364) with appropriate double label settings. The counting efficiencies were determined with an automatic external standard and quench correction curves. Counting efficiency for ^{3}H was 38-40.5% (mean 39%) and for ^{14}C, 62-64% (mean 63%). Corrections were applied for the overlap of counts between ^{3}H and ^{14}C channels; the ratio for $^{3}H/^{14}C$ being 0.0015 and for $^{14}C/^{3}H$, 0.29. Background counts were also subtracted.

(4) Purification of ^{14}C-d-Aldosterone:

4-^{14}C-d-Aldosterone was chromatographed in systems (v) and (iii) and then dissolved in benzene-methanol (1-1:v/v) to give approximately 4000cpm/ml prior to storage at 4°C. The purity was checked periodically in the same systems.

(5) Dilution, purification and specific activity determination of ^{3}H-acetic anhydride:

The specific activity of ^{3}H-acetic anhydride (500mCi/mM) was diluted to approximately 1mCi/mM with purified acetic anhydride according to the formula given by the Radiochemical Centre bulletin (1961): $W=Ma\ (1/A-1/A)$ where, W(mg) = weight of carrier compound added, M = molecular weight of compound, a(mCi) = total activity in the ampoule, A(mCi/mM) = molar specific activity of the compound and A'(mCi/mM) = molar specific activity of the

diluted compound. The diluted ^{3}H-acetic anhydride was distilled twice under reduced pressure (10-15mm Hg) and stored under nitrogen at -17°C.

The specific activity of the diluted ^{3}H-acetic anhydride was determined by acetylation of cortisol. Purified ^{3}H-acetic anhydride (50ul) and pyridine (20ul) were added to cortisol (200ug) and the reagents mixed in a tube that was tightly closed with a glass stopper moistened with a drop of pyridine and incubated at room temperature overnight. Following acetylation, 40% ethanol (1ml) was added and the mixture extracted with methylene chloride (5ml). The extract was washed with water (2ml), dried over anhydrous sodium sulfate and evaporated in a rotary-evapo-mix (Buchler Instruments). The product, cortisol-21-acetate, was purified by chromatography in systems (v), (vii) and (iii) and its concentration determined by the blue-tetrazolium reaction as described by Kliman and Peterson (1960) and Nowaczynski et al (1967). Briefly, the blank, standards and extracts were treated with blue-tetrazolium (0.1ml, 50mg/10ml absolute ethanol) and tetramethyl ammonium hydroxide (0.2ml, 100mg/10ml absolute ethanol), and each was diluted to 4ml with absolute ethanol. After mixing well, the reactants were kept for 1hr at room temperature in the dark, and the optical densities were measured at 525mu with a Unicam spectrophotometer (model SP-500). An aliquot was also taken for counting and the radioactivity expressed as dpm ^{3}H/ug cortisol. The result was multiplied by 2, since aldosterone forms a diacetate and cortisol a monacetate.

(6) Assay of urinary aldosterone

(i) Hydrolysis and extraction

Duplicate aliquots (25ml) of urine were adjusted to pH1 and an accurately known amount of ^{14}C-aldosterone (approximately 2500dpm) was added. The urines were incubated at room temperature for 24hr and the hydrolysates extracted with methylene chloride (3 x 2vol). The extracts were combined and

washed with ice-cold NaOH (0.1N, 1/10vol) and water (1/10vol). After drying over anhydrous sodium sulfate, the extracts were evaporated under reduced pressure and the residues transferred with benzene-methanol (1-1) into tubes and evaporated.

The dry extract was chromatographed in system (i) with aldosterone and cortisol as markers. The area corresponding to aldosterone was eluted, evaporated in a rotary-evapo-mix and dried completely in vacuum desiccator.

(ii) Acetylation

The dry residue was dissolved in pyridine (20ul) and reacted with ^{3}H-acetic anhydride (20ul). The tubes were closed with stoppers moistened with a drop of pyridine, and incubated at 37°C for 24hr in a desiccator containing conc. H_2SO_4 and solid KOH. Aldosterone-18-21-diacetate (30ug) and 40% ethanol (1ml) were added to each tube and the acetates extracted with methylene chloride (2 x 5ml). The extract was washed with water (2 x 1ml), dried over anhydrous sodium sulfate and evaporated.

(iii) Purification of aldosterone diacetate

The aldosterone-18,21-diacetate was purified sequantially by chromatography in systems (iv), (ii), (v), (vi) and (vii). After the final chromatography, the U-V band was eluted directly into a counting vial and evaporated. A distilled water blank (25ml) was included with each group of 10 samples and carried through the complete procedure.

(iv) Measurement of radioactivity

The dry residue was dissolved in 10ml toluene based scintillator (4gm PPO and 100mg POPOP/liter toluene) and counted with the appropriate double label settings. The counting efficiencies were determined as described above.

Calculations

ng aldosterone/25ml urine

$$= \frac{^{3}H \text{ cpm} \times \% \text{ Efficiency} \times \% \ ^{14}C \text{ Recovery}}{\text{specific activity of } ^{3}H\text{-acetic anhydride}}$$

Corrected ng aldosterone/25ml urine

= (ng aldosterone/25ml urine) - (ng ^{14}C-aldosterone + water blank)

ug aldosterone/25hr urine

$$= \frac{\text{Corrected ng aldosterone/25ml urine} \times \text{total urine vol.}}{1000 \times 25}$$

(v) Assessment of methodology

(a) Precision and reproducibility

The precision and reproducibility of the methodology were determinied by several estimations of aldosterone in the urine of two normal subjects. One urine assayed six times gave a mean of 14.3 ± 0.35 (S.D.) ug aldosterone/24hr (coefficient of variation (CV) 2.6%. The second urine, assayed nine times, gave a mean of 9.33 ± 0.45 (S.D.) ug aldosterone/24hr (C.V. 5.2%).

(b) Specificity

The specificity of the method was assessed by determining the $^{3}H/^{14}C$ ratio after each chromatography until a constant ratio was obtained. One urine gave the ratios 1.1, 0.51, 0.38, 0.35, 0.34 and 0.34 in systems (i), (iv), (ii), (v), (vi) and (vii) respectively. A further chromatography in system (viii) did not alter the ratio of 0.34. Since a constant $^{3}H/^{14}C$ ratio was obtained after system (v), samples were routinely chromatographed in systems (i), (iv), (ii), (v) and (vi).

(c) Recovery

Aldosterone (0.2 - 2.0ug) was added to water (50ml) and to urine (50ml) from an adrenalectomised patient. The samples were assayed as described above, and the recovery of aldosterone is given in Table 1. The mean recovery from both water and urine was 101.2%. The mean recovery of ^{14}C-d-aldosterone in 123 urines from normal and hypertensive subjects was 9.3% ± 1.81 (S.D.).

(d) Sensitivity

Aldosterone equivalents obtained with the non-specific water blank and urine from an adrenalectomised patient were not significantly different. The water blank gave a value of 14.0ng/25ml, (n=17, range 8-25ng) and the urine from the adrenalectomised patient 12.5ng/25ml compared with a range of 183-472ng/25ml urine for normal subjects. The values obtained for the water blank and the adrenalectomised patient's urine represent the lower limit of sensitivity of the method.

F. GLC Methodology

(1) Equipment

GLC was carried out on a Hewlett Packard High efficiency Dual column gas chromatograph (model 402), equipped with two hydrogen flame ionization detectors and a Hewlett Packard strip chart recorder (10", model 71271). U-shaped (6' x 4mm) glass columns were packed with 100/120 GCQ precoated with the liquid phases 3% OV-1, 3% SE-30, 3% XE-60, 2% XE-60 and 3% Hi-eff. 8BP. Acoiled (12' x 4mm) glass column was packed with 1% SE-30 on 100/120 GCQ. The column supports and glasswool were all silinized.

TABLE 1

Recovery of aldosterone from water and urine in the double isotope derivative assay

Sample	Aldosterone added (ug)	Aldosterone recovered (ug)	% Recovery
Water	0.2	0.211	105.5
	0.5	0.509	101.7
	1.0	0.981	98.1
	2.0	2.00	100.0
		Mean	101.2
Adrenalectomised patient's urine	0.2	0.198	99.0
	0.5	0.510	102.0
	1.0	1.011	101.1
	2.0	2.051	102.6
		Mean	101.2

(2) Conditions for GLC

All packed columns were conditioned with the detector detached, at 260°C for 1hr without a carrier gas flow, and then at 230°C for 48-72hr with a low carrier gas flow. Before running the samples, the columns were primed with a mixture of the TMSE derivatives of the compounds under study. The running conditions for each stationary phase is given in Table 2.

(3) Purity of steroid standards

The authenticity of steroid standards were checked by comparing melting points and chromatographic properties (TLC and GLC) with the literature values (Table 3).

(4) Internal standard and relative retention time

The retention time of a compound on a gas chromatogram is determined by the distance of the apex of its GLC peak from the point of injection. Since this value is subject to considerable variation with fluctuations in running condition, the GLC data may be expressed more meaningfully in terms of the relative retention time (RRT) of a compound compared to an internal standard.

The RRT is calculated from the formula:

$$RRT = \frac{\text{RT (cm) of compound}}{\text{RT (cm) of internal standard}}$$

The internal standard chosen should ideally have a retention time closely related to the compound(s) under study, but adequately separated to allow quantitation of the compound(s).

The RRT can be determined with high precision and is commonly used, although other methods of characterization of compounds on GLC, such as steroid number and methylene unit, have also been widely used (Wotiz and Clark, 1966; Eik-nes and Horning, 1968).

Table 2

Running conditions for GLC under isothermal operation (6' U shaped columns)

Conditions	Stationary phases					
	3% XE-60	3% Hi-eff-8BP	3% SE-30	3% OV-1	2% XE-60	1% SE-30*
Oven temp. (°C)	210	240	220	220	200	200
Flash heater (°C)	240	260	240	250	240	240
Detector (°C)	240	260	240	250	240	240
Nitrogen (ml/min)	40	40	50	50	50	50
Hydrogen (ml/min)	40	40	40	40	40	40
Air (ml.min)	300	300	300	300	300	300
Chart (inch/min)	0.25	0.25	0.25	0.25	0.25	0.25

*12'-coiled column

Table 3

Properties of reference steroids

Compound	MP (°C)	GLC RRT* Found	GLC RRT* Lit.	TLC Rf.** Found	TLC Rf.** Lit.
1. Androsterone	182-185	0.61	0.60	0.61	0.63
2. Etiocholanolone	152-155	0.71	0.71	0.55	0.53
3. DHEA	150-151	0.83	0.84	0.63	0.60
4. 11-keto androsterone	155-158	1.32	1.26	0.44	0.40
5. 11-keto etiocholanolone	189-190	1.53	1.47	0.35	0.33
6. 11β-hydroxyandrosterone	200-201	1.78	1.73	0.37	0.39
7. 11β-hydroxyetiocholanolone	234-237	2.19	2.12	0.31	0.34

*GLC as TMSE on 2% XE-60 with dihydrotestosterone as internal standard.

**TLC in systems (a) chloroform-ether (4-1) followed by
(b) ethylacetate-benzene (2-1)

Matthijssen and Goldzieher (1971)

(5) Derivative formation

Urinary extracts and standard steroids were taken in silylation tubes ("miniaktor") and thoroughly dried in a desiccator before adding the following reagents.

Trimethyl silylether (TMSE)

The 11-deoxy-17-KS were reacted with pyridine (0.75ml), hexamethyl-disilazane (0.2ml) and trimethylchlorosilane (10-15 drops). The 11-oxy-17-KS and 17-ketogenic steroids were reacted with "DMF-sil-Prep" (0.2ml). All reactions were carried out overnight at room temperature, and then the reactants were evaporated under a stream of nitrogen in a heating block at 40°C. The dried reaction products were dissolved in tetrahydrofuran (0.1ml), centrifuged and 1.5ul injected into the gas chromatograph.

(6) Identification of GLC peaks from urine

Several steroid peaks were observed in the GLC chromatograms of urinary extracts from normal and hypertensive subjects. The identity of each peak was established by comparing the relative retention time with a known standard (Table 4) on two different GLC phases. In addition, further evidence of identity was obtained by admixing the known standard steroid with the urinary extracts, and observing an increase in the peak area and height.

(7) Quantitation of GLC peaks

A progression curve was prepared with the TMSE derivatives of authentic steroids under study at concentrations approximating as closely as possible those of the samples. The peak area was calculated from peak height x width at half height, and the concentration of each steroid was then read directly from the progression curve. The following calculation was adapted from the reports of Cawley et al, 1967, and Marthijssen and Goldzieher, 1971).

Table 4

Relative retention times of standard 17-ketosteroids and urinary steroids after addition of authentic standards

Standard Steroids	Normal subjects						Hypertensive subjects			
	2% XE-60			3% Hi-eff-8BP			2% XE-60		3% Hi-eff-8BP	
	Std.	Urine	Urine + Std.	Std.	Urine	Urine + Std.	Urine	Urine + Std.	Urine	Urine + Std.
Androsterone*	0.60	0.61	0.60	0.56	0.56	0.56	0.60	0.60	0.57	0.57
Etiocholanolone*	0.71	0.72	0.72	0.76	0.76	0.76	0.72	0.72	0.76	0.76
Dehydroepiandrosterone*	0.84	0.84	0.84	0.90	0.90	0.90	0.85	0.85	0.90	0.90
11-ketoandrosterone*	1.32	1.33	1.32	1.29	1.29	1.29	1.33	1.33	1.29	1.30
11-ketoetiocholanolone*	1.55	1.54	1.55	1.70	1.69	1.69	1.54	1.54	1.68	1.68
11β-hydroxy androsterone*	1.80	1.81	1.81	2.18	2.18	2.18	1.81	1.81	2.17	2.17
11β-hydroxy etiocholanolone*	2.19	2.20	2.20	2.61	2.61	2.62	2.20	2.20	2.62	2.65
	3% SE-30			3%OV - 1			3% SE-30		3% OV-1	
Androstenedione**	0.65	0.65	0.65	0.66	0.66	0.66	0.65	0.65	0.65	0.65

*Dihydrotestosterone as internal standard; **Testosterone acetate as internal standard

$$\text{Each steroid (mg/24hr)} = \frac{\text{ug steroid (S) x injection factor (F) x Recovery (R) x urine aliquot}}{1000}$$

where, S = ug steroid calculated from peak area and progression curve

$$F = \frac{\text{vol of sample TMSE (ul)}}{\text{vol injected (ul)}}$$

$$R = \frac{100}{\text{\% recovery of radioactivity}}$$

G. GLC estimation of 11-deoxy-17-ketosteroids ($C_{19}O_2$-KS)

The conjugated 11-deoxy-17-ketosteroids in the urine were determined by GLC of their TMSE derivatives on 3% XE - 60, and involved a modification of the methods of Rivera et al (1967), Rivarola and Migeon (1966) and Bardin and Lipsett (1967).

(1) Purification of labelled steroids

1,2-^{3}H-etiocholanolone and 4-^{14}C-DHEA were purified by paper chromatography in the system ligroin-methanol-water (100-96-4:v/v/v) followed by TLC in the system toluene-methanol (97-3:v/v). The purified compounds were dissolved in benzene-methanol (1-1) to give a solution approximately 5000cpm of ^{14}C-DHEA and to 15000cpm of ^{3}H-etiocholanolone per ml, and each was stored at 4°C.

(2) Ketodase hydrolysis of urine

Duplicate aliquots (1/20) of 24hr urines and of a water blank were treated with 1ml of ^{14}C-DHEA (5000cpm) and ^{3}H-etiocholanolone (15000cpm) and the pH adjusted to 5. Acetate buffer (5ml, pH5, 2M) and Ketodase (800 units/ml) were added, and the mixture incubated at 37°C for 24hr. The hydrolysate was extracted with methylene chloride (3 x equal vol), and the combined organic phase washed with NaOH (2 x 1/10vol, 1N) followed by water (3 x 1/10 vol).

The alkaline and aqueous phases were combined and saved for solvolysis, and the clean extract dried over anhydrous sodium sulfate and evaporated under reduced pressure. This fraction contained the steroids liberated from the glucuronide conjugates.

(3) Solvolysis

Sulfate conjugates were solvolysed by the method of Burstein and Lieberman (1958). The pH of the combined aqueous phase was adjusted to 1 with H_2SO_4 (20%, v/v) after addition of sodium chloride (20gm/100ml), and to each was added ^{14}C-DHEA (5000cpm) and ^{3}H-etiocholanolone (15000cpm). After extraction with ethyl acetate (3 x ½vol), the combined extract was incubated at 37°C for 24hr, the solvolysate concentrated under pressure, and washed with NaOH (3 x 1/10vol, 1N) and water (3 x 1/10vol). The aqueous phases were pooled and extracted once with ethyl acetate, and the extract washed with water (2 x 1/10vol). The combined extract was dried over anhydrous sodium sulfate and evaporated under reduced pressure to give the sulfate conjugate fraction.

(4) Effect of Ketodase hydrolysis on DHEA-sulfate

The possibility that hydrolysis of DHEA-sulfate might occur during incubation with Ketodase, was investigated. ^{3}H-DHEA-sulfate was added to triplicate aliquots of urine, the urine extracted with methylene chloride (2 x equal vol) and the extract washed with water (2 x 1/10vol), dried over anhydrous sodium sulfate, and evaporated. The combined aqueous phase was sequentially hydrolysed with Ketodase and solvolysed in the usual manner. Aliquots of the corresponding hydrolysed fractions were counted. Only a small fraction (4.9%) of DHEA-sulfate was hydrolysed by Ketodase; the results of the hydrolyses were therefore not corrected for this quantity. The recovery of radioactivity in each fraction is given in Table 5.

(5) Silica gel chromatography

The glucuronide and sulfate fractions were purified and resolved further on silica gel (60-200mesh, 1gm). The silica gel was slurried in benzene and packed in glass columns (20 x 0.4cm). Dried extracts were applied in a few drops of benzene. Table 6 gives the elution sequence and recovery of ^{14}C-DHEA and ^{3}H-etiocholanolone after silica gel chromatography. These labelled steroids were added to all urines as tracer markers, and the second silica gel fraction was taken for GLC analysis of the 11-deoxy-17-ketosteroids.

(6) GLC estimation of androsterone, etiocholanolone and DHEA.

Androsterone, etiocholanolone and DHEA in the glucuronide and sulfate fractions were resolved as their TMSE derivatives on a 3% XE-60 column. The relative retention times with respect to the internal standard, cholesterol TMSE were 0.33, 0.38 and 0.45 respectively.

Fig. 2 illustrates the resolution of the TMSE derivatives of standard androsterone, etiocholanolone, DHEA and cholesterol on 3% XE-60, and Fig. 3 the progression curves for each compound in the range 0-2ug. Further chromatography on a 3% Hi-eff-8BP column indicated the absence of impurities.

The recovery of ^{14}C-DHEA and ^{3}H-etiocholanolone was determined for each experiment by counting a suitable aliquot before gas chromatography. Table 7 gives the overall mean recovery of ^{14}C-DHEA and ^{3}H-etiocholanolone in the sulfate and glucuronide fractions from 37 urines. Since labelled androsterone was not available commercially, its recovery was estimated from the average recovery of ^{3}H-etiocholanolone and ^{14}C-DHEA in each sample. The reproducibility of the methodology was determined by analysing a urine

Table 5

Recovery of radioactivity after ketodase hydrolysis of DHEA-sulfate

	% Recovery	
Fraction	Mean	Range
Free	12.1	11.5-13.3
Ketodase	4.9	4.8-5.1
Solvolysed	87.0	85.9-88.2

Table 6

Elution and recovery of ^{3}H-etiocholanolone and ^{14}C-DHEA on silica gel chromatography

Solvent	Fraction	% Recovery of Radioactivity			
		^{14}C-DHEA		^{3}H-etiocholanolone	
		mean	range	mean	range
(i) 10% ethyl acetate in benzene	1 (10ml)	0.3	0-0.3	0.45	0.4-0.5
(ii) 15% ethyl acetate in benzene	2 (25ml)	93.7*	90-95	82.5*	79.7-83.5
(iii) 15% ethyl acetate in benzene	3 (10ml)	0.55	0.4-0.6	3.2	2.0-4.0

*Fraction collected

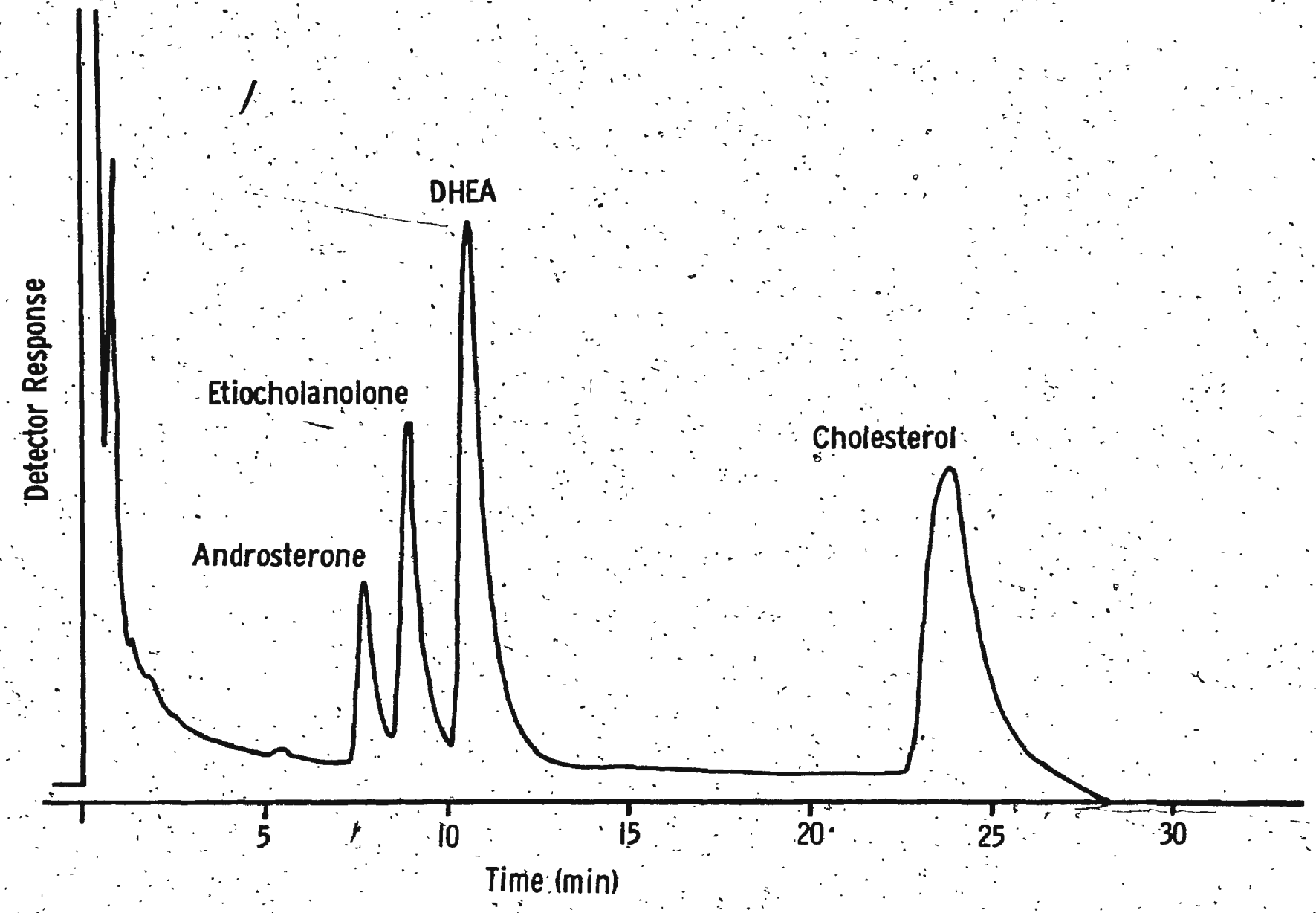


Fig. 2 Resolution of the TMSE derivatives of androsterone, etiocholanolone, DHEA and cholesterol on 3% XE-60

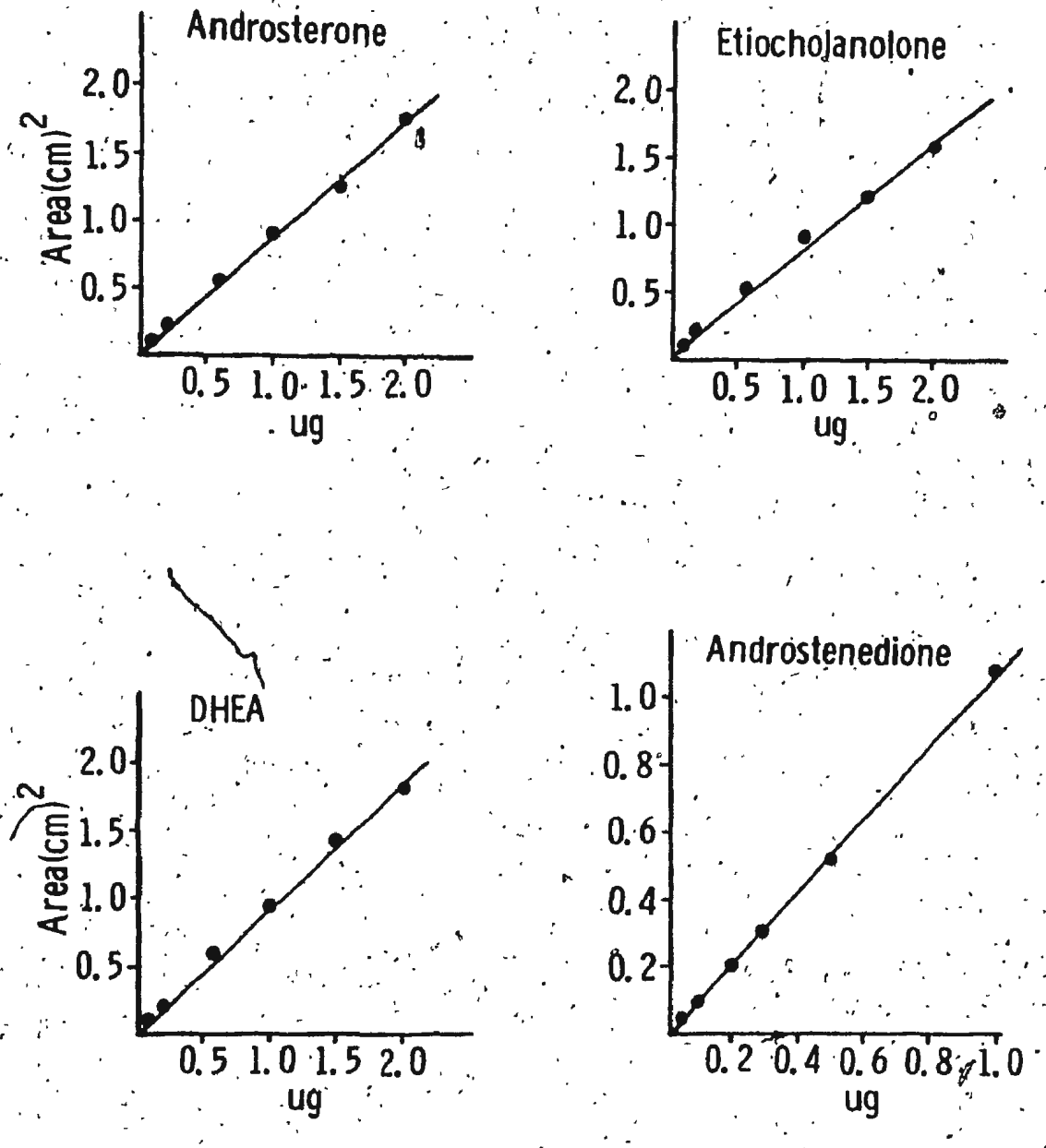


Fig. 3 Progression curves of standard androsterone, etiocholanolone and DHEA on 3% XE - 60, and androstenedione on 3% SE - 30

nine times for DHEA and etiocholanolone in the sulfate and glucuronide fractions. Table 8 gives the standard deviation and coefficient of variation for these estimations.

(7) GLC estimation of 4-androstene-3,17-dione

(i) Purification of 1,2-^{3}H-androstenedione

1,2-^{3}H-androstenedione was purified by paper chromatography in the system hexane-methanol-water (100-90-10:v/v/v), followed by the thin layer system benzene-ethyl acetate (60-40%v/v). The purified compound was dissolved in benzene-methanol (1-1) and diluted to approximately 5000cpm/ml.

(ii) Estimation of androstenedione

An aliquot of a 24hr urine (200ml) was extracted with diethyl ether-chloroform (3 x equal vol, 3-1:v/v). The combined extract was concentrated under reduced pressure and washed with NaOH (2 x 1/10vol, 1N) and water (3 x 1/10vol) to give a clean extract that was dried over anhydrous sodium sulfate and evaporated. The residue was purified consecutively by paper chromatography in the system hexane-methanol-water (100-90-10:v/v/v), followed by TLC in systems benzene-methanol (85-15:v/v) and benzene-ethyl acetate (60-40:v/v).

The area corresponding to Δ^{4}-A was eluted from the final chromatogram, dried thoroughly, dissolved in tetrahydrofuran (25ul) and an aliquot taken to determine the recovery of radioactivity. Fig. 4 gives the gas chromatogram of standard androstenedione on 3% SE-30. Further chromatography on 3% OV-1 confirmed the purity of the compound. The concentration of steroid in each peak was calculated from a progression curve (0.05-1ug) of standard androstenedione (Fig. 3). Δ^{4}-A had an RRT of 0.64 with respect to the internal standard testosterone acetate. The mean overall recovery of ^{3}H-androstenedione in the urine was 58.6%.

Table 7

Overall recovery of ^{3}H-etiocholanolone and ^{14}C-DHEA in urines*

	% (Mean) Recovery of radioactivity		
	A**	^{3}H-E	^{14}C-DHEA
Sulfate	57.0	57.5	56.5
Glucuronide	58.9	58.6	59.3

*17 normal and 20 hypertensive subjects

**Estimated from mean recovery of ^{3}H-E and ^{14}C-DHEA in each sample

Table 8

Reproducibility of the GLC estimation of etiocholanolone and DHEA in a representative urine*

	DHEA (mg/24hr)		Etiocholanolone (mg/24hr)	
	Sulfate	Glucuronide	Sulfate	Glucuronide
Range	1.9-2.07	0.43-0.50	0.48-0.64	0.44-0.52
Mean	2.0	0.46	0.58	0.47
±S.D.	0.05	0.02	0.03	0.03
% co-efficient of variation	2.6	5.0	4.8	7.0

*Analysed nine times

H. GLC estimation of 11-oxy-17-ketosteroids ($C_{19}O_3$-KS)

A spectrum of urinary 11-deoxy and 11-oxygenated 17-ketosteroids was resolved by GLC of their TMSE derivatives on 2% XE-60 with a procedure modified from the reports of Faucette and Cawley (1971) and Matthijssen and Goldzieher (1971).

(1) Purification of labelled steroids

The purification of 4-^{14}C-DHEA was carried out as previously described. 1,2-^{3}H-tetrahydrocortisol was purified by paper chromatography in the systems benzene-hexane (1-1:v/v)-formamide and benzene-methanol-water (4-2-1:v/v) and then dissolved in ethanol to give approximately 10,000cpm/ml.

(2) Preparation of ^{3}H-11β-hydroxy-etiocholanolone

Since labelled 11β-hydroxy-etiocholanolone was not available commerically, it was synthesised from 1,2-^{3}H-tetrahydrocortisol. Tetrahydrocortisol (200ug) was mixed with ^{3}H-tetrahydrocortisol (30Ci/mM) and oxidised with periodic acid as described by Bush (1961). Briefly, the steroid was dissolved in methanol (0.5ml) and reacted with periodic acid solution (5ml, 4gm/100ml of 0.2N H_2SO_4). The reactants were incubated in the dark at room temperature for 18hr, and the product extracted with ethanol (2 x 2ml) after the addition of conc. ammonium hydroxide (0.1ml). The supernatant obtained by centrifugation was evaporated and purified in several chromatographic systems as indicated in Table 9. TLC plates were developed in system (a) and after drying, re-run in the same direction in system (b). Standard 11β-hydroxy etiocholanolone and tetrahydrocortisol were also run as markers and visualised with iodine vapour, and the corresponding 11β-hydroxy etiocholanolone band was further purified in systems (c-e) after removal

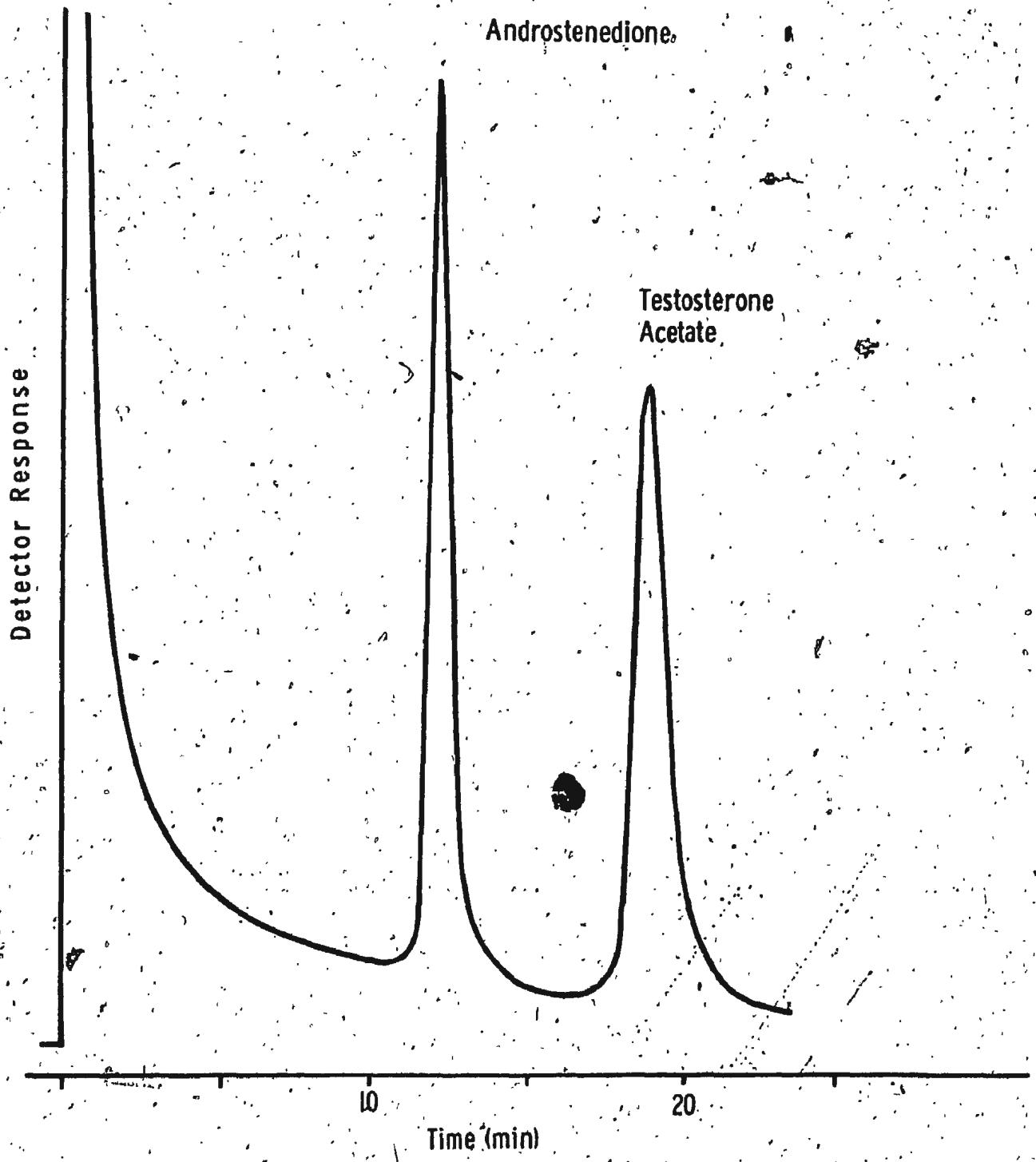


Fig. 4 Gas chromatogram of androstenedione on 3% SE-30

from the TLC plates. The concentration of the product was determined by the Zimmerman reaction as previously described for the colorimetric estimation of total 17-ketosteroids and an aliquot was counted for radioactivity. The specific activity of the product was 10,010dpm/ng).

(3) Amberlite XAD-2 resin chromatography

Amberlite XAD-2 resin (10gm) was washed three times with distilled water until the finer particles and chloride ions were removed. The resin was packed as a slurry in glass columns (150 x 9mm) to give a flow rate of 3-4ml/min. One ml of ^{14}C-DHEA (5000cpm), ^{3}H-11β-hydroxy etiocholanolone (5000cpm) and ^{3}H-THF (10,000cpm) standard solutions were combined and concentrated before addition to an aliquot of urine (10ml). The urine was percolated through the Amberlite resin, washed with water (50ml) and the steroid conjugate fraction eluted with methanol (50ml) and evaporated under reduced pressure. Table 10 gives the elution sequence and recovery of radioactivity.

(4) Hydrolysis of urine

The methanol eluate from the Amberlite column was evaporated and the residue dissolved in acetate buffer (15ml, pH5, 2M) and hydrolysed with glusulase (0.1ml/10ml urine) for 48hr at 37°C. The hydrolysate was extracted with methylene chloride (3 x equal vol) and the combined extracts washed with NaOH (1 x 1/10vol, 0.1N) and water (2 x 1/10vol). The extract was dried over anhydrous sodium sulfate and evaporated under reduced pressure.

(5) Silica gel chromatography

Silica gel (2g, 100-200mesh) was slurried in 20% ethyl acetate in ligroin and packed in glass columns (18 x 0.7cm). The dry residue from the hydrolysate extract was applied to the column in a few drops of the same solvent and elution carried out as given in Table 11. The mean recoveries

Table 9

Purification of synthesised ^{3}H-11β-hydroxyetiocholanolone

	System	Running time
(a) *TLC:	Chloroform-ether (4-1:v/v)	40min
(b) *TLC:	Ethyl acetate-benzene (2-1:v/v)	40min
(c) **Paper:	Benzene-methanol-water (2-1-1:v/v/v)	8hr
(d) **Paper:	Heptane-toluene-methanol water (2-1-2,55-0.45:v/v/v/v)	8hr
(e) ***TLC:	Benzene-ethyl acetate (40-60:v/v)	40min

*Matthijssen and Goldzieher (1971)

**Roberts et al (1968)

***Kirschner & Lipsett (1964)

Table 10

Elution and recovery of ^{14}C-DHEA, ^{3}H-etiocholanolone and ^{3}H-tetrahydrocortisol from Amberlite XAD-2 chromatography

Fraction	% Recovery of radioactivity			
	^{14}C-DHEA		^{3}H-E + ^{3}H-THF	
	mean	range	mean	range
Urine (10ml)	2.1	2.1-2.2	2.2	2.0-2.6
Water (50ml)	2.3	2.1-2.5	2.6	2.0-3.0
Methanol (50ml)	90.4*	86.3-95.0	91.2*	88.8-03.8

*Fraction collected

of the three major steroid fractions, $C_{19}O_2$ and $C_{19}O_3$ metabolites and the corticosteroids (C_{21}) are also given. Fractions (ii) and (iii) were combined and the components estimated as their TMSE derivatives. Fraction (iv) was converted to 17-ketogenic steroids before GLC (see Later).

(6) GLC estimation of 11-deoxy and 11-oxy-17-ketosteroid fractions

Several chromatographic systems were investigated in order to obtain the best conditions for the chromatography of the TMSE derivatives of the $C_{19}O_2$ and $C_{19}O_3$ steroids. The best resolution was effected on a 2% XE - 60 column under isothermal conditions.

Fig. 5 gives the resolution of the TMSE derivatives of the standards, androsterone, etiocholanolone, dehydroepiandrosterone, dihydrotestosterone, 11-keto androsterone, 11-keto etiocholanolone, 11β-hydroxy androsterone, and 11β-hydroxy etiocholanolone. The RRT for each compound with respect to dihydrotestosterone (internal standard) is given in Table 12. This Table also gives the RRT's of the five commercially available C-3 epimers of A, E, DHEA, KA and HA; namely isoandrosterone, 3β-hydroxyetiocholanolone, 5-androsten-3α-ol-17-one, 11-ketoisoandrosterone and 11β-hydroxyisoandrosterone respectively.

Fig. 6 gives the progression curve plotted for each standard in the range 0.1 - 2.0ug.

The mean recoveries of $C_{19}O_2$ and $C_{19}O_3$ compounds, determined from the recovery of ^{14}C-DHEA and ^{3}H-11β-hydroxy etiocholanolone, were 76.8% and 77.3% respectively.

I. GLC estimation of 17-ketogenic steroids

In order to facilitate the GLC analysis of the corticosteroid fractions they were converted to 17-ketosteroids. These compounds were then termed 17-ketogenic steroids.

Table 11

Elution and recovery of 17-ketosteroids and corticosteroids from silica gel columns

Fractions	% Recovery of radioactivity					
	$C_{19}O_2$*		$C_{19}O_3$**		C_{21}***	
	mean	range	mean	range	mean	range
(i) 20% ethyl acetate in ligroin (20ml)	1.9	0.71-3.0	0.12	0.1-0.24	0	0
(ii) 40% ethyl acetate in ligroin (25ml)	94.8****	88.9-102	1.2	0.56-1.9	0	0
(iii) 60% ethyl acetate in ligroin (25ml)	1.4	1.2-1.6	87.1****	82.2-96.3	10.0	9.5-10.4
(iv) 100% ethyl acetate (25ml)	2.0	1.5-3.1	6.2	5.8-6.9	93.1****	92.1-93.9

* Recovery of ^{14}C-DHEA

** Recovery of ^{3}H-11β-hydroxyetiocholanolone

*** Recovery of ^{3}H-tetrahydrocortisol

**** Fraction collected

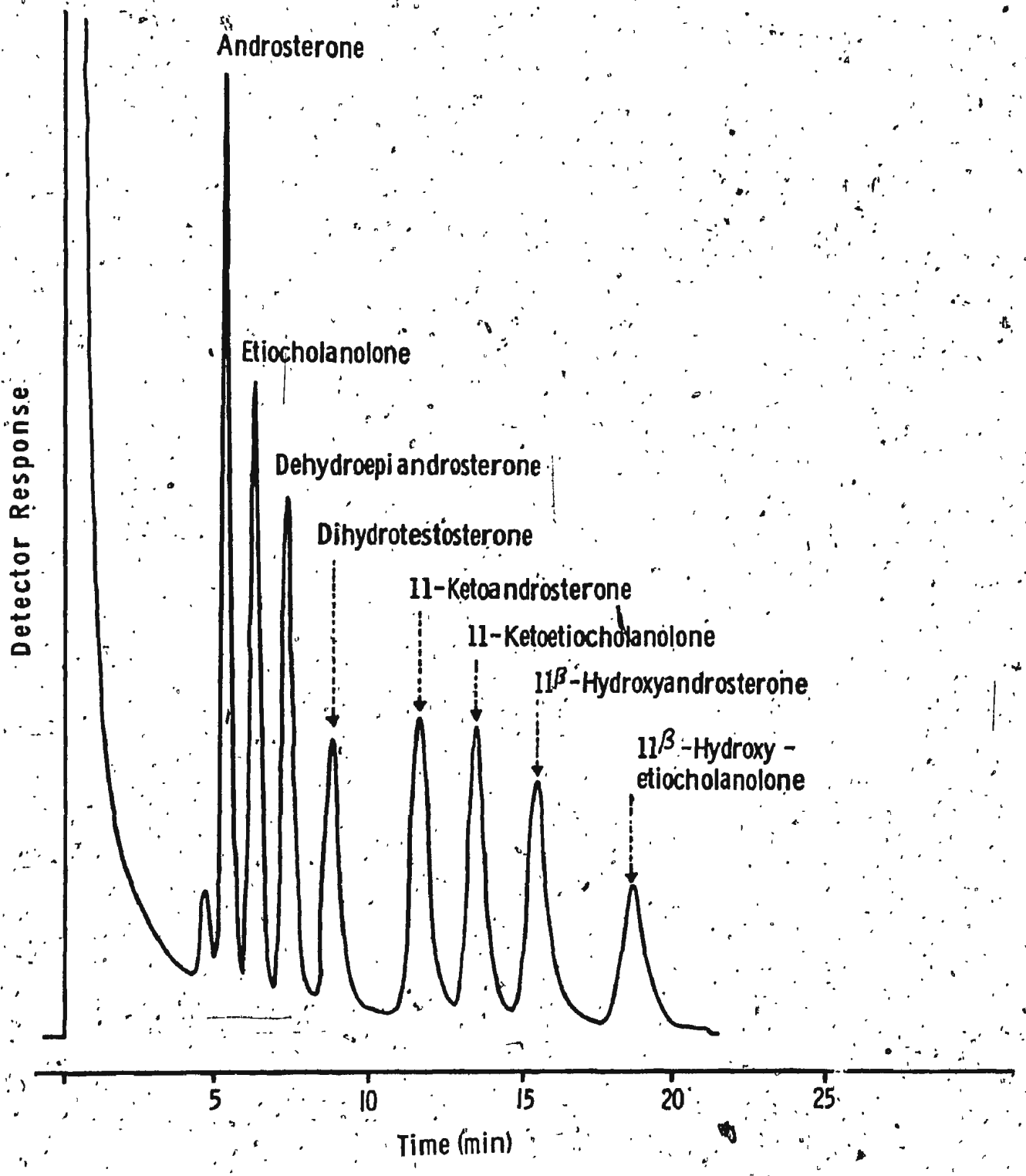


Fig. 5 Resolution of the TMSE derivatives of the 11-deoxy plus 11-Oxy-17-ketosteroid standards on 2% XE-60

Table 12

Relative retention times of standard 11-deoxy and 11-oxy-17-ketosteroids on 2% XE - 60

Compound	RRT with respect to dihydrotestosterone
Androsterone	0.61
Etiocholanolone	0.71
DHEA	0.83
11-keto-androsterone	1.32
11-keto-etiocholanolone	1.53
11β-hydroxyandrosterone	1.78
11β-hydroxyetiocholanolone	2.19
Isoandrosterone	0.91
3β-hydroxyetiocholanolone	0.58
5-androsten-3α-ol-17-one	0.56
11-keto-isoandrosterone	2.5
11β-hydroxyisoandrosterone	3.0

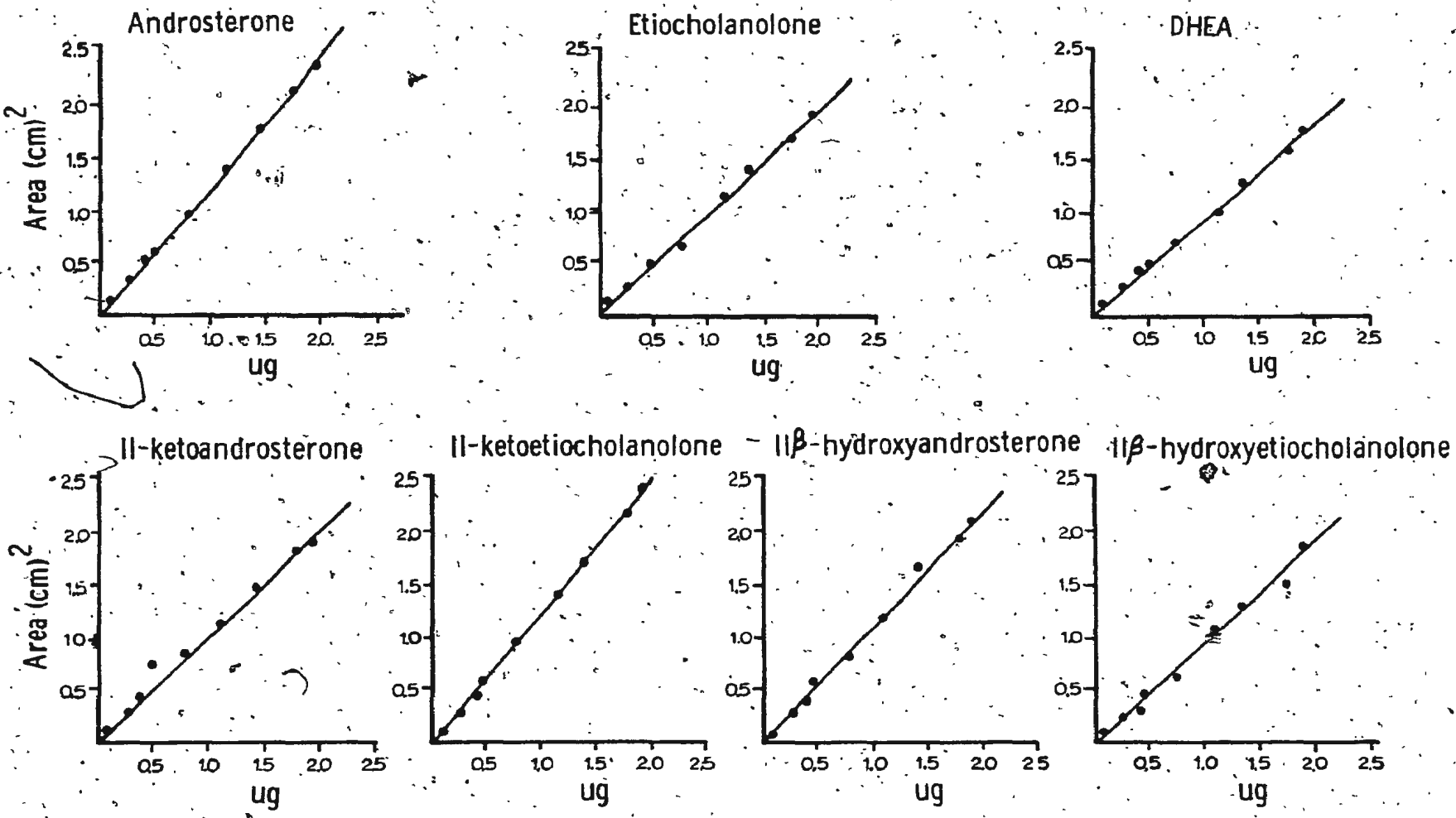


Fig. 6 Progression curves of standard 11-deoxy and 11-oxy-17-ketosteroid on 2% XE-60

(1) Steroid side chain cleavage and extraction

The corticosteroid fraction (C_{21}) isolated by silica gel column chromatography was reduced with sodium borohydride and oxidised with sodium periodate to give the 17-ketogenic fraction essentially as described by Edwards (1964) and Makin (1970). Briefly, the fraction was dissolved in a few drops of ethanol, diluted with water (5ml) and the pH adjusted to 7. Sodium borohydride (2ml, 10% solution in 0.01N NaOH) was added and the mixture incubated at 50°C for 24min. An acid-acetone mixture (2ml, glacial acetic acid-acetone-water: 1-1-2:v/v/v) was added and the mixture again incubated for 20min at 50°C. Ether was added as required to avoid frothing, and the pH readjusted to 7 before the addition of an aqueous solution of sodium periodate (8ml, 10%). The mixture was incubated further for 40min at 50°C, cooled, and extracted with methylene chloride (3 x 20ml). The combined methylene chloride extract was washed with NaOH (1 x 1/10vol, 0.1N) and water (2 x 1/10vol), dried over anhydrous sodium sulfate and evaporated under reduced pressure. The reaction product contained three major 17-keto-steroids derived from the C_{21}-steroids as given in Table 13.

(2) GLC estimation of etiocholanolone, 11β-hydroxy androsterone and 11β-hydroxy etiocholanolone

These three compounds were estimated as their TMSE derivatives on 2% XE - 60. The resolution, RRT and the progression curve of these steroid standards have been described previously. (Table 12; Figs. 5 and 6)

The mean recovery of the corticosteroids (17-ketogenic steroids), determined from the recovery of ^{3}H-tetrahydro cortisol, was 78.1%.

3. HYPERTENSIVE AND NORMAL SUBJECTS

All hypertensive subjects studied were inhabitants of four Newfoundland communities, (Fogo, Ramea, Badger and Bay de Verde) who took part in a blood pressure survey in this province (Abbott et al, (1971), a,b).

The normal subjects studied for 11-deoxy-17-ketosteroids were also from the same four Newfoundland communities, whereas for the 11-oxy-17-keto-steroids and 17-ketogenic steroids, they were normal, healthy laboratory personnel.

The hypertensive and normal subjects in the Newfoundland communities chosen for urinary studies were examined to exclude secondary causes of hypertension. Briefly, they had a casual diastolic pressure greater than 90mm of mercury, a finding which was confirmed on subsequent clinical reassessment. All were examined in their communities with special attention paid to family history of hypertension, ocular fundi, heart, peripheral pulses, abdominal bruits and for clues indicating the presence of coarctation of the aorta, Cushing's Syndrome and pheochromocytoma. The cardiac, renal and cerebrovascular systems were assessed for evidence of deterioration due to hypertension. No subjects had thyrotoxicosis, diabetes mellitus, or were known to have a carcinoma, and all were untreated and on unrestricted diets.

Laboratory tests were performed on the urines from all subjects at the General Hospital, St. John's. The urinalysis, sodium, potassium, and vanillyl mandelic acid (VMA) were within normal limits except for the following individuals: F8 (N) had an albumin value of 2+, F62 (H) had a sugar of 1+, and the VMA values for F6 (N), F66 (H), F64 (H), F69 (H) and R54 (H), were 9.9, 16.9, 13.3, 11.5, and 10.9mg/24hr respectively. The VMA values for those subjects were above the upper limit of the assay (normal

Table 13

Possible origin of 17-ketosteroids obtained by reduction-oxidation of C_{21}-steroid fraction

C_{21}-steroid	17-ketosteroid produced
Pregnanetriol Tetrahydro-11-deoxycortisol	Etiocholanolone
Tetrahydrocortisone Tetrahydrocortisol 5β-cortol 5β-cortolone	11β-hydroxyetiocholanolone
allo-tetrahydrocortisol 5α-cortol 5α-cortolone	11β-hydroxyandrosterone

values range up to 9mg/24hr), but since the subjects were on unrestricted diets, the significance of these results is uncertain.

Single 24hr urines were collected from both groups in polyethylene bottles without preservative and stored frozen at -20°C until processed.

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Section I

Classification of subjects - general laboratory investigations

(i) Classification - blood pressure

The blood pressures of 55 individuals in the four Newfoundland communities of Fogo, Ramea, Badger and Bay de Verde and 15 normal laboratory personnel were measured with a sphygmomanometer (London School of Hygiene and Tropical Medicine). Subjects were designated hypertensive or normal on the basis of their blood pressure together with other medical considerations, including family history, medical history, age, etc.* The subjects in each community were designated by a number and the code F, R, B or V to represent their community as mentioned above. The normal laboratory personnel were designated as M1-7 and F1-8 for males and females respectively.

Table 14 indicates that all normal subjects had a diastolic pressure below 86mm Hg (range 65-86). The systolic pressure of these subjects ranged from 108-178mm Hg with 26 subjects having values below 137. In the hypertensive group (Table 15) 36 subjects had a diastolic pressure above 90 with one at 89 (range 89-128). Systolic pressures ranged from 127-224mm Hg with 32 subjects above 140 and 5 below.

(ii) Age and sex of subjects

Twenty-eight females and 9 males were included in the hypertensive group, and 20 females and 13 males in the group of subjects with normal blood pressures. The normal subjects ranged in age from 21-78 years with 28 subjects under 60 years and 5 over. Among the normal male and female subjects, two males and three females were over 60 years.

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*I am indebted to Dr. E.C. Abbott for the blood pressure measurements and the classification of subjects.

The ages of the hypertensive subjects ranged from 25-69 years with 32 subjects under 60 years and 5 over. In this group two male and three female subjects were over 60 years.

(iii) Urine volume and creatinine excretion

Complete collection of 24hr urines was assumed from both the total volume of urine voided and the creatinine excretion. Tables 14 and 15 indicate that the normal subjects had a lower mean creatinine excretion (1.1g/24hr) than the hypertensive subjects (1.24g/24hr); however, the difference was not statistically significant. Several of the normal and hypertensive subjects excreted low levels of creatinine, which may indicate differences in the body mass or a failure to collect all the urine voided. However, no significant difference was evident in the mean volume of urine voided by the two groups.

(iv) Aldosterone excretion

The excretion of aldosterone was estimated in the 24hr urines by a double isotope derivative assay method. All the subjects were on unrestricted diets and freely ambulatory. Table 16 gives the results of individual normal and hypertensive subjects. The range of aldosterone excretion in the group of normal subjects was 1.1 - 20.4ug/24hr (mean 10.14ug) and in the group of hypertensive subjects, 2.6 - 18.7ug/24hr (mean 9.63ug). There was no significant difference between the two groups in the mean excretion of aldosterone, whether expressed as ug/24hr urine or ug/g creatinine. These results ruled out the possibility of primary aldosteronism in the subjects studied.

Table 14

Classification of NORMAL subjects, and the excretion of urine and creatinine

Code	Blood Pressure	Age	Wt. lbs.	Urine vol. (ml/24hr)	Creatinine (g/24hr)
		Males			
F7	134/79	39	150	1000	1.68
F14	147/75	59	128	1550	1.47
F41	126/80	25	173	1700	1.20
B45	137/65	32	141	1160	2.18
V46	178/83	73	164	945	0.90
F53	170/81	68	173	3000	1.87
M1*	130/80	33	180	1220	1.69
M2	128/80	47	160	1460	1.95
M3	124/84	28	143	820	1.41
M4	126/80	42	154	880	1.84
M5	114/70	32	120	1880	1.43
M6	120/70	26	150	2760	2.31
M7	132/86	31	150	2730	1.57
		Females			
F10	149/79	47	187	1030	1.62
B50	137/81	32	159	1320	1.40
V8	109/76	72	120	745	0.68
B52	147/77	51	153	680	1.34
V17	133/66	47	147	1680	0.51
V39	129/78	78	92	1210	0.80
B32	158/84	59	115	700	0.80
V3	115/65	28	145	1330	1.06
V14	129/68	26	126	1710	1.47
B1	163/75	67	143	1050	0.92
V7	116/76	28	116	965	1.03
F1*	120/65	33	125	780	1.25
F2	118/74	26	120	1320	1.29
F3	108/74	30	127	760	0.83
F4	122/75	31	140	730	1.24
F5	124/80	41	130	1060	1.10
F6	128/78	21	120	740	0.89
F7	124/76	29	98	1100	0.60
F8	122/78	30	125	1880	1.12
B34	89/65	27	118	1090	0.51

CREATININE EXCRETION
(Total Population)

Mean	1.27
SD±	0.45
SE±	0.08
P**	N.S.***

*Normal laboratory personnel

Student 't' test, P : Differences between the total population of Normal and Hypertensive subjects

***Not significant where P is greater than 0.05

Table 15

Classification of HYPERTENSIVE subjects, and the excretion of urine and creatinine

Code	Blood Pressure	Age	Wt. lbs.	Urine Vol. (ml/24hr)	Creatinine (g/24hr)
		Males			
F26	117/94	68	181	1690	1.84
B35	213/101	57	194	800	2.10
R15	152/98	43	135	1660	1.59
F6	146/97	36	207	1340	2.39
F59	135/96	40	179	770	2.23
F60	154/95	50	135	1700	0.39
F65	157/90	32	136	1230	1.94
F66	171/92	42	170	2020	0.70
V44	214/107	63	108	1590	0.55
V57	139/91	54	164	1570	1.33
V27	176/102	50	146	800	1.05
F1	186/107	39	177	1230	0.71
B25	224/126	41	222	1120	2.00
V31	194/112	53	149	1225	1.00
B61	163/95	52	180	1070	1.60
F68	216/114	54	118	1560	1.00
R78	141/91	57	150	1760	1.64
V47	219/128	60	150	915	1.14
F28	167/107	42	199	910	1.40
F22	146/96	39	155	880	1.69
F47	157/93	41	106	780	0.62
B38	185/105	64	174	1090	1.50
R12	149/114	42	144	940	1.27
R52	137/94	47	210	1460	1.60
B12	210/110	54	106	760	0.42
B59	150/95	50	216	900	0.53
B64	166/98	52	207	1000	0.83
F61	136/93	43	168	1280	1.32
F62	171/99	47	194	1560	1.16
F63	159/96	53	198	1460	0.47
F64	194/92	42	130	2220	1.41
F69	171/89	25	217	1320	1.94
R72	127/94	33	176	1680	1.25
V32	214/120	53	160	675	0.51
V42	140/96	52	180	1140	0.70
V59	187/97	62	148	1570	1.48
R54	201/124	69	153	1460	2.10

CREATININE EXCRETION
(Total Population)

Mean	1.24
SD±	0.57
SE±	0.09
P**	N.S.

P**: Differences between the total population of Normal and Hypertensive subjects

Section II

Studies on the 11-deoxy-17-ketosteroids excreted as sulfate and glucuronide conjugates in the urine of NORMAL and HYPERTENSIVE subjects

(i) GLC resolution of the 11-deoxy-17-ketosteroid spectrum

The 11-deoxy-17-ketosteroid fractions were isolated from the urines of normal and hypertensive subjects as described in the Methodology section. The components of the glucuronide and sulfate conjugate fractions were extracted after hydrolysis and resolved as their TMSE derivatives by GLC. Fig. 7 compares the resolution of the three major 11-deoxy-17-ketosteroids, androsterone (A), etiocholanolone (E), and dehydroepiandrosterone (DHEA) in the two conjugate fractions isolated from the urine of normal and hypertensive subjects. The spectra illustrate a satisfactory resolution of the steroids with negligible overlap of peaks. In particular there is an absence of peaks that would interfere with the quantitation of the steroids under study.

(ii) Excretion of the individual 11-deoxy-17-ketosteroids in the conjugate fractions

Tables 17 and 18 give the individual values for the excretion of A, E and DHEA in the glucuronide and sulfate fractions isolated from the urine of normal and hypertensive subjects. The total excretion of these compounds is also given by the summation of the two conjugate fractions. The group of 17-ketosteroids as measured colorimetrically, is compared with the summations of A+E+DHEA measured by GLC.

Table 16

Excretion of aldosterone in the urine of NORMAL and HYPERTENSIVE subjects

Normal Subjects				Hypertensive Subjects					
Aldosterone (ug/24hr)				Aldosterone (ug/24hr)					
Males		Females		Males		Females			
Code		Code		Code		Code		Code	
F7	8.4	F10	6.4	F26	15.0	V57	18.2	F62	9.2
FI4	14.3	B50	11.0	B35	12.0	V27	14.8	F63	2.7
F41	12.0	V8	12.2	R15	8.3	F1	15.2	F64	8.0
F45	9.5	B52	1.1	F6	6.0	B25	7.6	F69	14.0
V46	4.5	V17	17.4	F59	18.7	V31	11.3	R72	9.8
F53	13.9	V39	14.5	F60	9.3	B61	12.2	V32	7.7
*M1	13.8	B32	9.8	F65	11.6	F68	10.0	V42	10.6
M2	20.4	V3	10.1	F66	9.2	R78	3.5	V59	9.2
M3	17.9	V14	5.4	V44	3.6	V47	4.8	R54	3.7
M4	9.3	B1	8.4			F28	5.8		
M5	13.9	V7	7.3			F22	2.6		
M6	10.7	F1	7.6			F47	5.2		
M7	8.1	F2	10.3			B38	11.7		
		F3	7.9			R12	14.0		
		F4	6.7			R52	14.2		
		F5	6.6			B12	8.6		
		F6	6.4			B59	7.8		
		F7	14.8			B64	10.9		
		F8	5.1			F61	9.4		
		B34	8.9						

Total Population			Total Population		
	ug/24hr	ug/g creatinine		ug/24hr	ug/g creatinine
Mean	10.14	9.77	Mean	9.63	11.75
±SD	4.17	4.30	±SD	4.10	6.75
±SE	0.74	1.11	±SE	0.68	1.68
			P	N.S.	N.S.

(iii) Comparison of differences in the excretion of the 11-deoxy-17-ketosteroids in the urine of normal and hypertensive subjects

Table 19 gives the mean excretion of the three major 17-ketosteroids in the sulfate and glucuronide fractions isolated from the urine of normal and hypertensive subjects and the significance of the differences between the two groups. In Table 20 the significance of the differences in the excretion of the 11-deoxy-17-ketosteroids when expressed as mg/24hr and as mg/g creatinine is given. The latter was included to ensure that the steroid values given in mg/24hr were related to complete and accurate collections of 24hr urines.

The results of the excretion of the three major steroids may be summarised as follows:

Dehydroepiandrosterone

The level of DHEA-S was highly significantly reduced in the urine of the hypertensive subjects ($P<0.001$), and although the excretion of DHEA-G was not altered, the total quantity of DHEA (G+S) excreted was significantly reduced ($P<0.001$) due to the effect of DHEA-S.

Etiocholanolone

The excretion of E-G in the urine of the hypertensive population was markedly reduced ($P<0.001$), but no significant difference was found for E-S. The total excretion of E (G+S) was also markedly reduced in the hypertensive group ($P<0.001$) and reflects the reduced excretion of E in the glucuronide fraction.

Androsterone

The mean excretion of A-G was found to be significantly increased ($P<0.01$) in the urine of the hypertensive subjects when the values were expressed as mg/24hr, but no significant difference was evident when the results were given as mg/g creatinine. Furthermore, no difference was found in the excretion of A-S in the total amount of A (G+S).

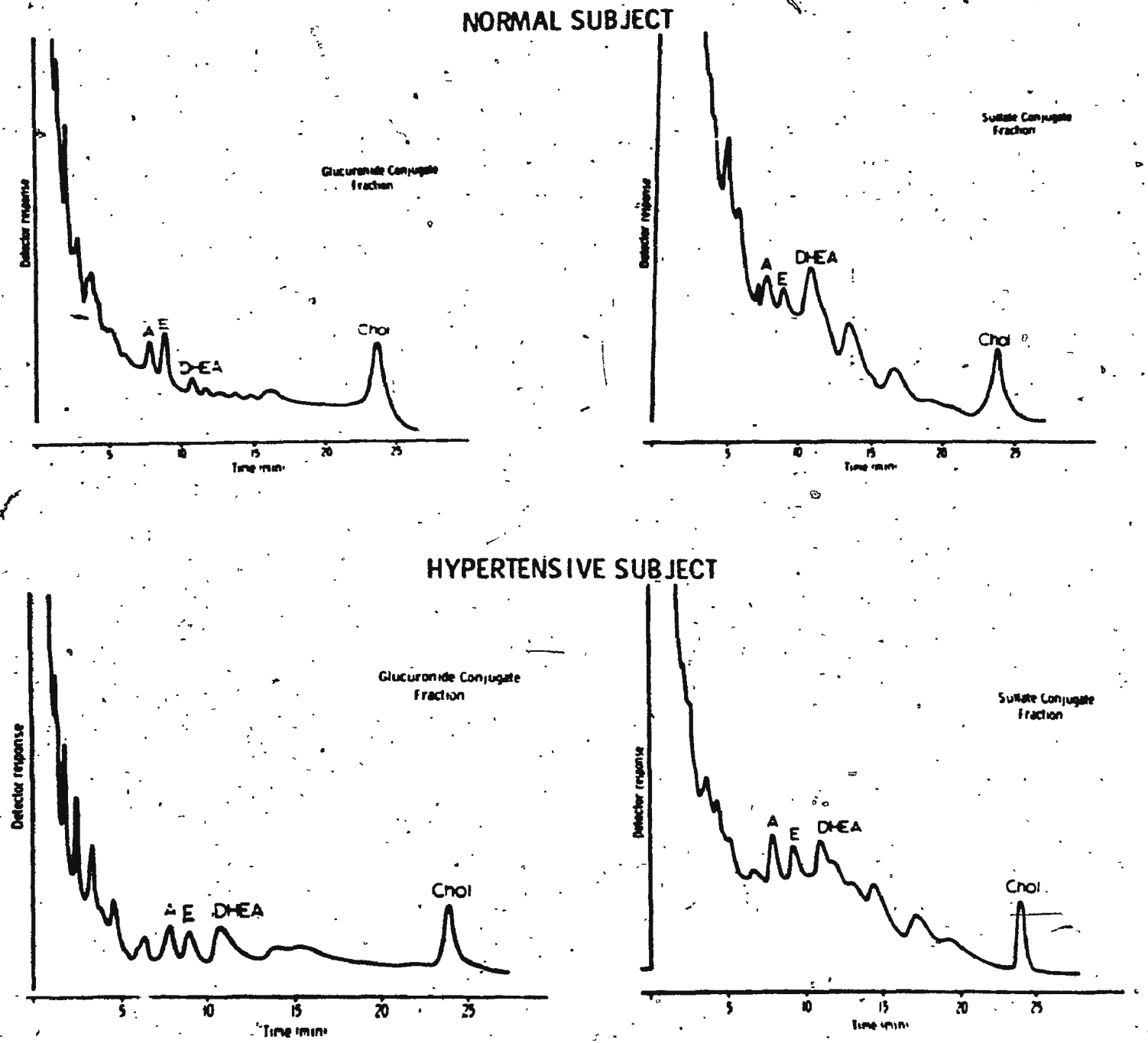


Fig. 7 GLC resolution of the 11-deoxy-17-ketosteroids in the conjugate fractions isolated from the urine of NORMAL and HYPERTENSIVE subjects

Table 17

Excretion of total 17-ketosteroids and the glucuronide (G) and sulfate (S) conjugates of androsterone, etiocholanolone and DHEA (mg/24hr) in the urine of NORMAL subjects

	A			E			DHEA			Total		Total*
Code	G	S	G+S	G	S	G+S)	G	S	G+S	A+E	A+E+DHEA	17-KS
Males												
F7	0.23	2.10	2.33	1.51	0.14	1.65	0.25	1.97	2.22	3.98	6.20	4.60
F14	0.14	1.58	1.72	2.10	0.12	2.22	0.31	2.58	2.89	3.94	6.83	-
F41	0.72	1.25	1.97	1.75	0.18	1.93	0.16	4.92	5.08	3.90	8.98	10.7
B45	0.40	1.95	2.35	1.20	0.12	1.32	0.11	1.38	1.49	3.67	5.16	7.4
V46	0.25	1.37	1.67	1.73	0.09	1.82	0.27	2.94	3.21	3.44	6.65	4.2
F53	1.10	0.75	1.85	2.55	0.11	2.66	0.28	3.50	3.78	4.51	8.29	10.7
Females												
V17	0.81	1.66	2.47	1.56	0.21	1.77	0.31	3.00	3.31	4.24	7.55	4.7
V39	0.56	1.78	2.34	0.91	0.09	1.00	0.11	5.11	5.22	3.34	8.56	4.0
B32	0.15	2.09	2.24	1.80	0.15	1.95	0.60	4.52	5.12	4.19	9.31	4.4
V3	0.16	1.95	2.11	0.54	0.05	0.59	0.06	3.74	3.80	2.79	6.50	8.7
V14	0.61	1.58	2.19	1.97	0.20	2.17	0.27	3.18	3.45	4.36	7.81	12.3
B1	0.27	1.58	1.85	2.53	0.09	2.62	0.33	2.50	2.83	4.47	7.30	3.8
V7	0.76	1.92	2.68	0.88	0.14	1.02	0.19	3.0	3.19	3.71	6.89	10.5
F10	0.63	2.33	2.96	1.60	0.08	1.68	0.21	2.20	2.41	4.64	7.05	12.0
B50	0.26	2.18	2.44	0.47	0.06	0.53	0.06	3.50	3.56	2.97	6.53	8.7
V8	0.91	1.98	2.89	1.40	0.10	1.50	0.09	1.58	1.68	4.39	6.07	3.8
B52	0.77	1.52	2.29	1.78	0.12	1.90	0.11	2.62	2.73	4.19	6.92	3.2

*Colorimetric assay

Table 18

Excretion of total 17-ketosteroids and glucuronide (G) and sulfate (S) conjugates of androsterone, etiocholanolone and DHEA (mg/24hr) in the urine of HYPERTENSIVE subjects

	A			E			DHEA			Total	Total	Total*
Code	G	S	G+S	G	S	G+S	G	S	G+S	A+E	A+E+DHEA	17-KS
Males												
F26	0.10	1.43	1.53	0.42	0.13	0.55	0.40	0.10	0.50	2.08	2.58	5.0
B35	0.70	2.40	3.10	0.48	0.13	0.61	0.22	0.18	0.40	3.71	4.11	4.8
R15	0.70	2.45	3.15	0.21	0.39	0.60	0.71	0.13	0.84	3.75	4.59	6.4
F6	0.36	1.65	2.01	0.78	0.23	1.01	0.70	0.15	0.85	3.02	3.87	8.6
F59	1.61	1.50	3.11	0.98	0.18	1.16	0.11	0.11	0.22	4.27	4.49	4.8
Females												
V47	0.74	1.99	2.73	1.20	0.18	1.38	0.19	1.20	1.39	4.11	5.50	7.9
F28	0.55	1.33	1.88	0.68	0.17	0.85	0.27	3.38	3.65	2.73	6.38	6.5
F22	0.67	1.35	2.02	0.20	0.99	1.19	0.25	0.01	0.34	3.21	3.55	6.2
F47	1.20	2.02	3.22	0.38	0.13	0.51	0.19	0.12	0.31	3.73	4.04	7.9
B38	1.85	1.34	3.19	0.36	0.11	0.47	0.43	0.09	0.52	3.66	4.18	4.7
R12	0.47	1.65	2.12	0.44	0.11	0.55	0.25	0.10	0.35	2.67	3.02	5.5
R52	1.42	1.55	2.97	0.33	0.21	0.54	0.08	0.18	0.26	3.51	3.77	9.9
V57	0.36	1.55	1.91	0.05	0.25	0.30	0.17	0.16	0.33	2.21	2.54	8.7
V27	1.42	3.06	4.48	1.42	0.16	1.58	0.18	0.22	0.40	6.06	6.46	5.6
F1	0.81	2.33	3.14	0.22	0.09	0.31	0.19	0.17	0.36	3.45	3.81	3.3
B25	0.59	1.25	1.84	0.92	0.12	1.04	0.15	0.18	0.33	2.88	3.21	6.2
V31	0.90	1.81	2.71	1.50	0.20	1.70	0.20	0.12	0.32	4.41	4.73	3.8
B61	1.46	1.48	2.94	0.56	0.26	0.82	0.30	0.08	0.38	3.76	4.14	6.4
F68	1.87	1.52	3.39	0.32	0.30	0.62	0.16	0.22	0.38	4.01	4.39	2.4
R78	0.87	1.09	1.96	1.81	0.19	2.00	0.24	2.40	2.64	3.96	6.60	3.6

*Colorimetric assay

Ratio of 5α/5β compounds

The A/E ratio, which gives a measure of the relative 5α/5β reductase activity in the metabolism of the 11-deoxy-17-ketosteroids was determined in the glucuronide, sulfate and total fractions. A significantly increased ratio was found for the hypertensive population in both the glucuronide and glucuronide plus sulfate fractions ($P<0.001$) as a result of a marked reduction in the excretion of etiocholanolone. In the sulfate fraction, by contrast an increased ratio was found in the normal group ($P<0.02$); however both hypertensive and normal subjects excreted low levels of the 5β- reduced compound (E) and comparable amounts of the 5α-compound (A) as sulfate conjugates.

Total 17-ketosteroids

No significant difference was found in the excretion of the total 17-ketosteroids, as measured colorimetrically, in the two groups of subjects studied. In addition, the values for the mean excretion of total 17-ketosteroids failed to correlate well with A+E+DHEA (G+S) measured by GLC. The latter was highly significantly reduced in the hypertensive group ($P<0.001$).

(iv) Releative 5α- and 5β-reductase activity

So far the excretion of the 5α-(androsterone) and 5β-reduced (etiocholanolone) steroids by the hypertensive and normal subjects have been compared. In Table 21 the relative proportions of these metabolites have been compared within the normal group and also within the hypertensive group to obtain an indication of the relative steroid reductase activities in each group. The results may be summarised as follows:

Normal subjects

The normal subjects excreted more etiocholanolone than androsterone in the glucuronide fraction (in the proportions of 3:1), which suggests a greater 5β-reductase activity.

Table 19

Differences in the excretion of conjugated 11-deoxy-17-KS (mg/24hr) in the urine of NORMAL and HYPERTENSIVE subjects

Steroids		Total population Mean	±SD	±SE	P value	Males Mean	±SD	±SE	P value	Females Mean	±SD	±SE	P value
Glucuronides													
A	N	0.51	0.29	0.07		0.47	0.33	0.08		0.54	0.26	0.10	
	H	0.93	0.50	0.11	< 0.01	0.69	0.51	0.25	N.S.	1.01	0.47	0.13	< 0.05
E	N	1.49	0.61	0.15		1.81	0.42	0.20		1.40	0.61	0.21	
	H	0.66	0.48	0.11	< 0.001	0.57	0.27	0.14	< 0.001	0.69	0.52	0.14	< 0.01
DHEA*	N	0.22	0.13	0.03		0.23	0.08	0.03		0.21	0.15	0.05	
	H	0.38	0.49	0.04	N.S.	0.43	0.24	0.12	N.S.	0.36	0.55	0.02	N.S.
Sulfates													
A	N	1.74	0.38	0.09		1.50	0.45	0.16		1.87	0.25	0.12	
	H	1.74	0.48	0.11	N.S.	1.89	0.44	0.22	N.S.	1.69	0.48	0.13	N.S.
E	N	0.12	0.04	0.01		0.13	0.03	0.01		0.12	0.05	0.01	
	H	0.22	0.19	0.04	N.S.	0.21	0.09	0.05	N.S.	0.23	0.21	0.05	N.S.
DHEA*	N	3.07	1.04	0.26		2.88	1.13	0.44		3.18	0.97	0.32	
	H	0.46	0.85	0.20	< 0.001	0.13	0.03	0.01	< 0.001	0.57	0.96	0.26	< 0.001
Glucuronides plus sulfates													
A	N	2.25	0.37	0.09		1.97	0.28	0.17		2.14	0.33	0.10	
	H	2.67	0.71	0.16	N.S.	2.58	0.68	0.34	N.S.	2.70	0.72	0.19	N.S.
E	N	1.67	0.60	0.15		1.93	0.42	0.20		1.52	0.63	0.22	
	H	0.89	0.47	0.11	< 0.001	0.79	0.25	0.12	< 0.001	0.92	0.52	0.14	< 0.05
DHEA*	N	3.29	1.07	0.27		3.11	1.14	0.43		3.39	1.01	0.34	
	H	0.74	0.86	0.20	< 0.001	0.56	0.25	0.12	< 0.001	0.79	0.97	0.26	< 0.001
A+E	N	3.92	0.52	0.13		3.91	0.33	0.19		3.93	0.61	0.18	
	H	3.56	0.86	0.20	N.S.	3.37	0.75	0.38	N.S.	3.62	0.88	0.23	N.S.
A+E+DHEA	N	7.21	1.06	0.26		7.03	1.27	0.43		7.32	0.91	0.31	
	H	4.28	1.08	0.26	< 0.001	3.93	0.72	0.36	< 0.001	4.45	1.24	0.33	< 0.001

*Level of significance did not alter after correcting results for the mean 4.9% DHEA-S estimated to be hydrolysed by Ketodase hydrolysis (cfp-51)

....2

Steroids		Total population Mean	±SD	±SE	P value	Males Mean	±SD	±SE	P value	Females Mean	±SD	±SE	P value
A/E Ratios*	N	0.45	0.22	0.04		0.35	0.19	0.08		0.50	0.23	0.06	
	H	2.37	2.03	0.45	< 0.001	1.43	1.23	0.55	< 0.01	2.69	2.18	0.56	< 0.01
Sulfates	N	17.12	9.53	2.31		12.23	4.27	1.74		19.79	10.67	3.22	
	H	10.43	5.86	1.31	< 0.02	10.24	4.89	2.19	N.S.	10.50	6.30	1.63	< 0.02
G+S	N	1.69	1.07	0.26		1.10	0.42	0.17		2.02	1.19	0.36	
	H	3.94	2.35	0.52	< 0.001	3.55	1.50	0.67	< 0.01	4.07	2.60	0.67	< 0.02
17-KS**	N	7.11	3.29	0.84		3.15	7.52	5.00		6.61	3.34	0.80	
	H	5.91	1.92	0.44	N.S.	1.61	5.96	5.00	N.S.	5.91	2.05	0.41	N.S.

*Mean values calculated from individual ratios

**Colorimetric assay

Table 20

Level of significance of differences* in the excretion of conjugated 17-ketosteroids expressed as mg/g creatinine**, and as mg/24hr, in the urine of NORMAL and HYPERTENSIVE subjects

Steroids	Total population		Males		Females	
	mg/g Creatinine	mg/24hr Urine	mg/g Creatinine	mg/24hr Urine	mg/g Creatinine	mg/24hr Urine
Glucuronides						
A	N.S.	0,01	N.S.	N.S.	N.S.	0.05
E	0.001	0.001	0.001	0.001	0.01	0.01
DHEA	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Sulfates						
A	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
E	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
DHEA	0.001	0.001	0.01	0.001	0.001	0.001
Glucuronides plus sulfates						
A	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
E	0.001	0.001	0.001	0.001	0.01	0.05
DHEA	0.001	0.001	0.01	0.001	0.001	0.001
A+E	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
A+E+DHEA	0.001	0.001	0.01	0.001	0.001	0.001
Steroid ratios						
A/E	0.01	0.001	0.01	0.01	0.05	0.02
Total 17-KS**	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

*Student 't' test' P<

**Colorimetric assay

By contrast, significantly less etiocholanolone than androsterone was excreted in the sulfate fraction (1:4), suggesting that the 5β-reductase activity is less important than the 5α-reductase activity. As a result of these differences the total excretion of A (G+S) was only slightly higher than E (G+S) and the difference was not statistically significant.

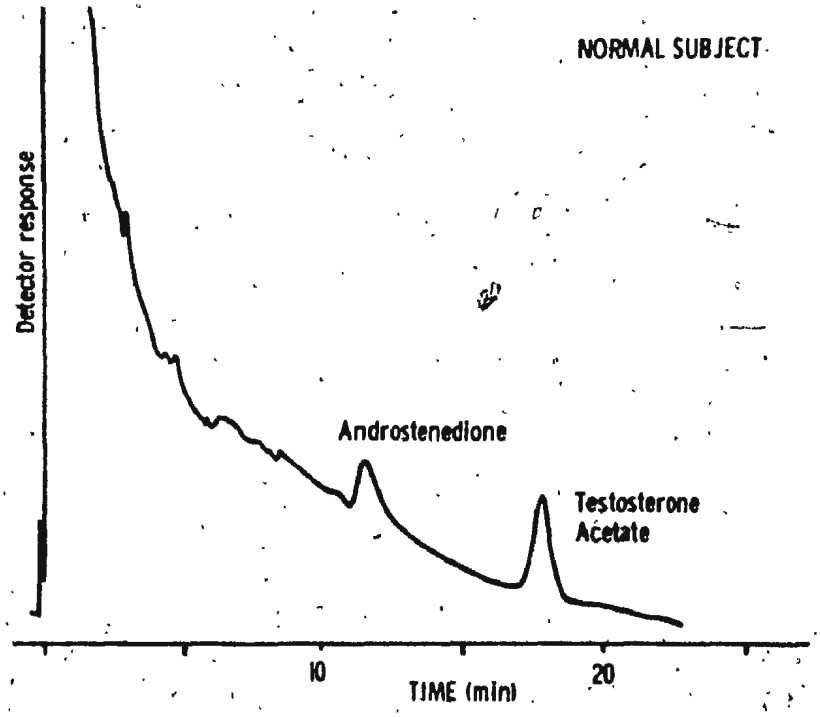
Hypertensive subjects

The hypertensive subjects excreted less etiocholanolone than androsterone in both glucuronide and sulfate fractions, but only the excretion of E-S was significantly different.

Comparison of the proportions of A and E excreted in the urine of the normal subjects studied and in the urine of the hypertensive subjects, revealed that similar proportions were excreted by both groups after sulfate formation. However, whereas more of the 5β-compound, etiocholanolone, was excreted as a glucuronide than androsterone by the normal subjects, the reverse was true for the hypertensives. Since the excretion of E-S was markedly reduced in the hypertensive group the total excretion, E (G+S) was also significantly less.

(v) Excretion of androstenedione

Androstenedione, which is an intermediate in the formation of A and E, was measured in order to determine whether alterations in the excretion of these latter compounds was related to the metabolism of Δ^4-A. Since Δ^4-A is excreted as a free steroid in low concentration, it was isolated separtely. Fig. 8 gives the gas chromatogram of this compound after isolation from the urine of a normal and a hypertensive subject.



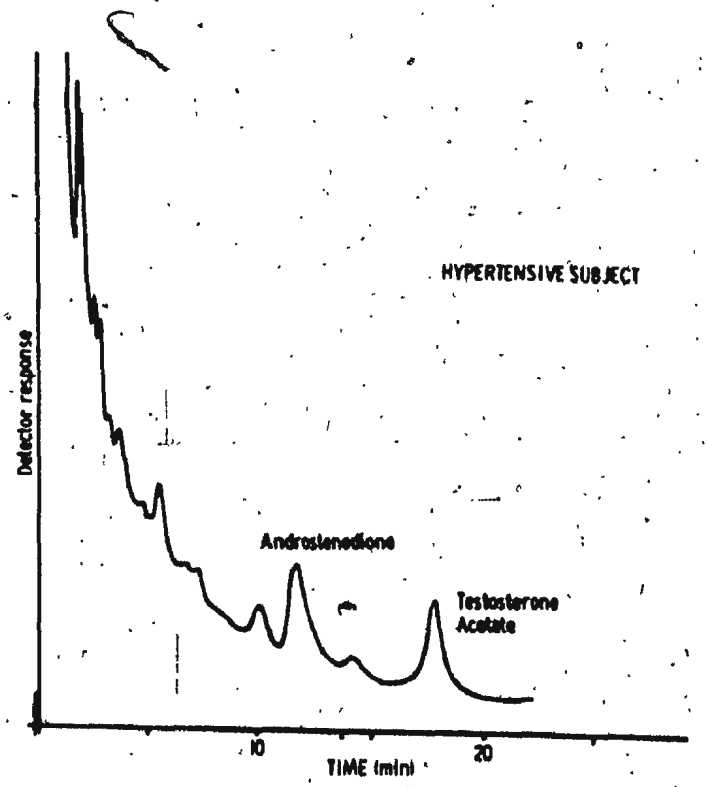


Fig. 8 GLC resolution of androstenedione isolated from the urine of NORMAL and HYPERTENSIVE subjects

Table 21

Differences in the mean excretion of 5α- and 5β- reduced 17-ketosteroids (mg/24hr) in the urine of NORMAL and of HYPERTENSIVE subjects

Steroids	Total population Mean	±SD	±SE	P value	Males Mean	±SD	±SE	P value	Females Mean	±SD	±SE	P value
Normal subjects												
Glucuronides												
A	0.51	0.29	0.07		0.47	0.33	0.15		0.53	0.26	0.08	
E	1.54	0.58	0.14	< 0.001	1.80	0.43	0.19	< 0.001	1.40	0.61	0.19	< 0.001
Sulfates												
A	1.74	0.38	0.09		1.50	0.45	0.20		1.87	0.25	0.08	
E	0.12	0.03	0	< 0.001	0.12	0	0	< 0.001	0.11	0.04	0	< 0.001
Glucuronides plus sulfates												
A	2.25	0.37	0.09		1.97	0.28	0.12		2.40	0.31	0.10	
E	1.66	0.60	0.15	N.S.	1.93	0.42	0.19	N.S.	1.52	0.63	0.20	N.S.
Hypertensive subjects												
Glucuronides												
A	0.93	0.50	0.11		0.69	0.51	0.25		1.01	0.47	0.12	
E	0.66	0.48	0.11	N.S.	0.57	0.27	0.13	N.S.	0.69	0.53	0.14	N.S.
Sulfates												
A	1.74	0.48	0.11		1.88	0.44	0.22		1.69	0.48	0.12	
E	0.22	0.19	0.03	< 0.001	0.21	0.09	0.04	< 0.001	0.23	0.21	0.05	< 0.001
Glucuronides plus sulfates												
A	2.67	0.71	0.17		2.58	0.68	0.34		2.70	0.72	0.19	
E	0.89	0.47	0.10	< 0.001	0.78	0.25	0.12	< 0.001	0.92	0.31	0.14	< 0.001

Individual Δ^4-A are given in Table 22. The mean excretion of Δ^4-A expressed as ug/24hr urine and ug/g creatinine was higher in the hypertensive group, but only the results expressed as ug/24hr urine were statistically significant ($P<0.01$). Females predominated in both groups, and no attempt was made to analyse the results of males and females separately.

(vi) Effect of age on the excretion of 11-deoxy-17-ketosteroids

The effect of age on the excretion of A, E and DHEA (G+S) in the urine of normal and hypertensive subjects is illustrated in Fig. 9. In each case a regression curve is drawn from the scatter plot. It is evident from this that no significant decline occurred in the subjects investigated in the present study. Neither did blood pressure have a significant influence on the values. DHEA and E, which were excreted at low levels by the hypertensive subjects, remained consistently low between the ages of 25-69.

(vii) Effect of blood pressure on the excretion of 11-deoxy-17-ketosteroids

The effects of increasing blood pressure on the excretion of the 11-deoxy-17-ketosteroids, was examined by plotting the individual steroid values against increasing blood pressure. Fig. 10 gives the relationship between the diastolic and Fig. 11 the systolic blood pressure and the excretion of DHEA, A and E by normal and hypertensive subjects.

No definite correlation between these parameters was evident apart from DHEA. The majority of the subjects with diastolic pressures above 90mm Hg excreted low levels of DHEA; however several individuals were atypical.

Table 22

Excretion of androstenedione (ug/24hr) in the urine of NORMAL and HYPERTENSIVE subjects

Normal subjects*		Hypertensive subjects**	
Code	Δ^4-A	Code	Δ^4-A
F7	1.8	B35	1.3
B50	5.9	B15	8.2
B45	4.0	F6	5.1
V46	1.4	F59	4.8
V17	1.8	B38	1.7
V39	1.9	R12	2.4
B32	0.23	R52	7.3
V3	2.3	V57	4.3
V14	2.5	V27	7.6
B1	3.1	F1	6.3
V7	1.6	B25	4.8
V8	1.7	V31	4.8
B32	1.2	B61	1.3
		F68	1.5
		R78	2.7

Total Population		Total Population	
ug/24hr	ug/g Creatinine	ug/24hr	ug/g Creatinine
Mean 2.26	2.07	Mean 4.16	2.90
±SD 1.37	1.09	±SD 2.27	1.84
±SE 0.39	0.31	±SE 0.58	0.49
		P < 0.01	N.S.

*3 males and 10 females

**4 males and 11 females

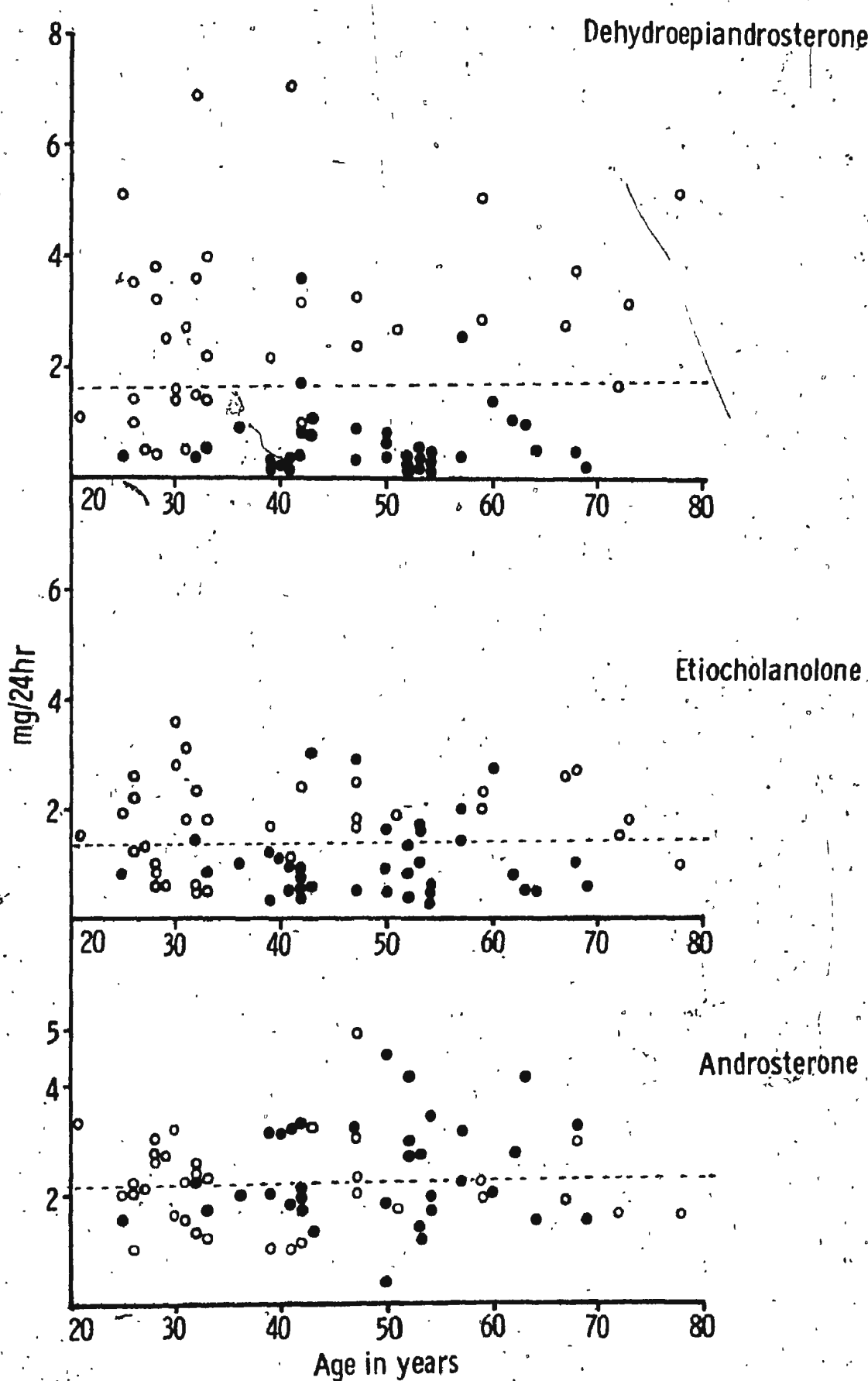


Fig. 9 Excretion of 11-deoxy-17-ketosteroids in the urine of NORMAL and HYPERTENSIVE subjects with increasing age

Normal subjects ○

Hypertensive subjects ●

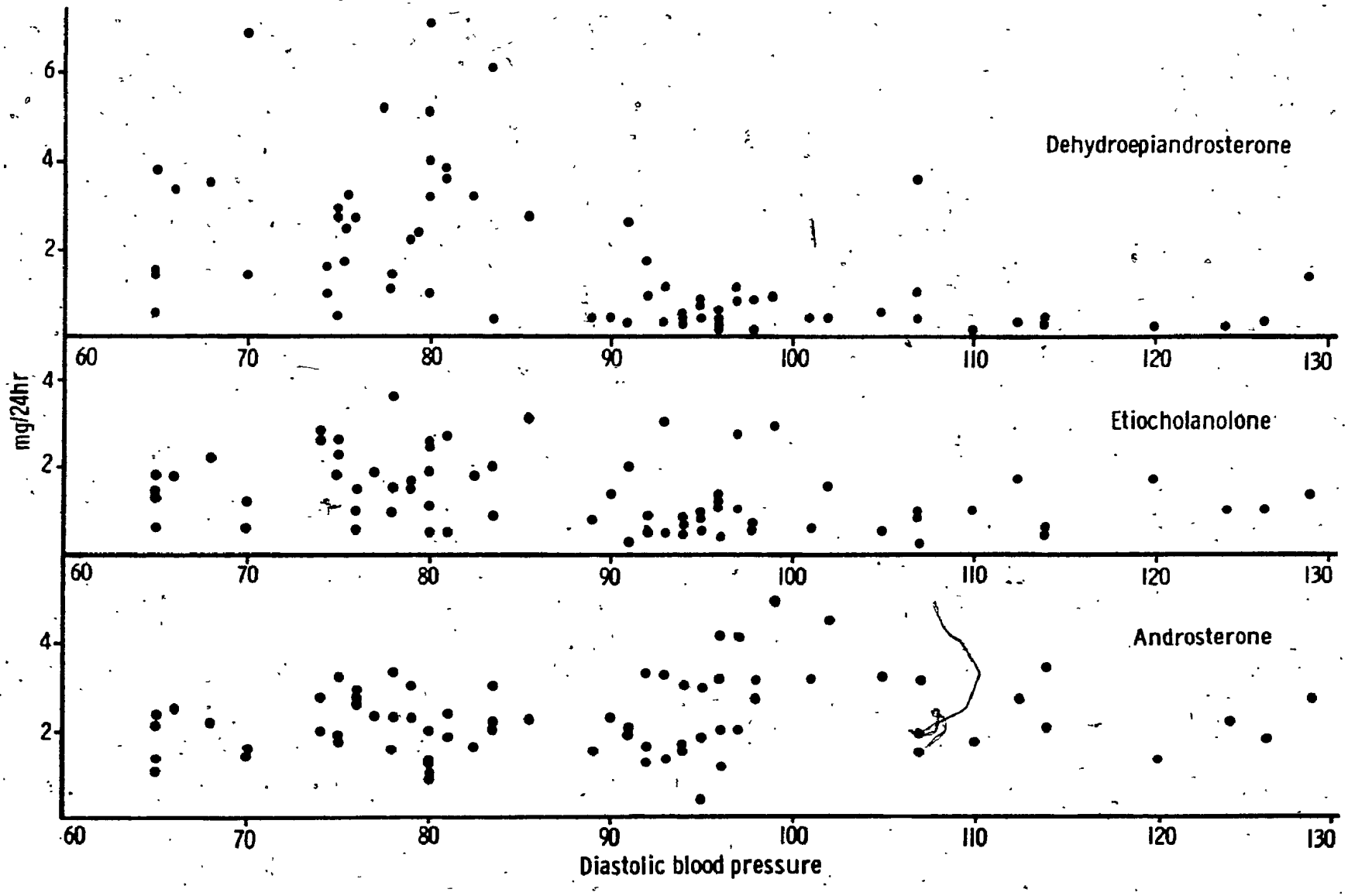


Fig. 10 Excretion of 11-deoxy-17-ketosteroids in the urine of NORMAL and HYPERTENSIVE subjects with increasing diastolic blood pressure

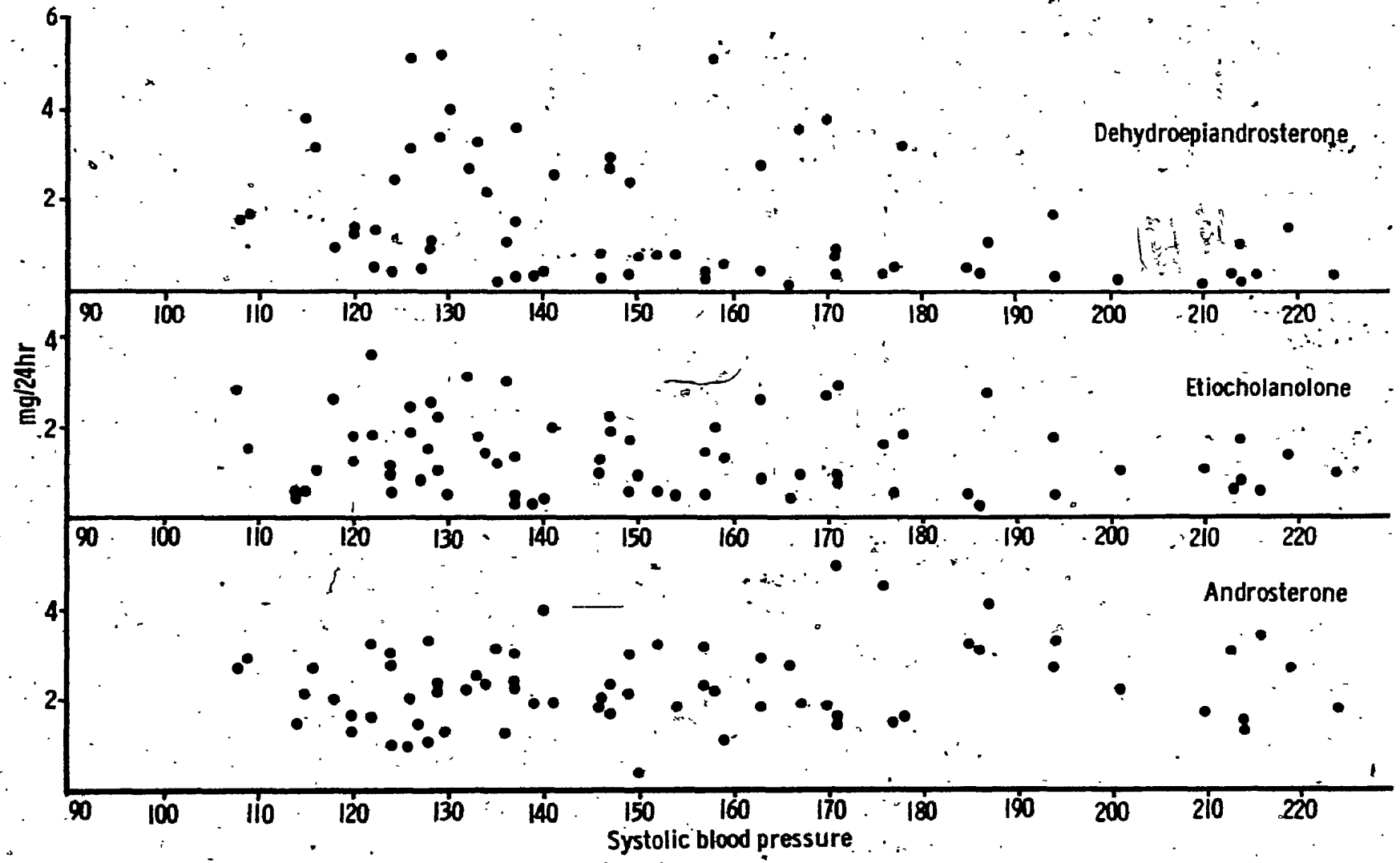


Fig. 11 Excretion of 11-deoxy-17-ketosteroids in the urine of NORMAL and HYPERTENSIVE subjects with increasing systolic blood pressure

Section III

Studies on the 11-deoxy and 11-oxy-17-ketosteroids excreted in the urine of NORMAL and HYPERTENSIVE subjects

(i) GLC Resolution

The 11-oxy-17-ketosteroids ($C_{19}O_3$) were analysed in the urines of a separate group of normal and hypertensive subjects to that studied in the previous section. In order to compare these two sets of subjects the 11-deoxy-17-ketosteroids ($C_{19}O_2$) were also chromatographed. Fig. 12 gives the resolution of the seven major metabolites quantitated in the $C_{19}O_2$ and $C_{19}O_3$ steroid fractions isolated from the urine of a hypertensive and normal subject.

The spectra illustrate a satisfactory resolution of the steroids and the absence of other peaks which interfere with the quantitation of the steroids under study.

(ii) Excretion of the individual 11-deoxy- and 11-oxy-17-ketosteroids

Tables 23 and 24 give the individual values for the excretion of A, E and DHEA in the $C_{19}O_2$ fraction and KA, KE, HA, HE in the $C_{19}O_3$ fraction isolated from the urine of normal and hypertensive subjects. The total excretion of A+E+DHEA and of KA+KE+HA+HE is also given. The group analysis of 17-ketosteroids is compared with the total values of $C_{19}O_2$ and $C_{19}O_3$-KS as measured by GLC.

(iii) Comparison of the differences in the excretion of 11-deoxy- and 11-oxy-17-ketosteroids in the urine of normal and hypertensive subjects

Table 25 compares the mean excretion of the seven major 17-ketosteroids isolated from the urine of normal and hypertensive subjects and the significance of the differences between the two groups.

The difference found in the excretion of A, E and DHEA confirmed the results in the previous section.

The results of the excretion of the 11-oxy-17-ketosteroids were as follows:

11-ketoandrosterone

The excretion of KA was higher (1.61mg) in the hypertensive group than the normal group (0.65mg), and the difference was statistically significant ($P<0.01$).

11-ketoetiocholanolone, 11β-hydroxyandrosterone and 11β-hydroxy-etiocholanolone

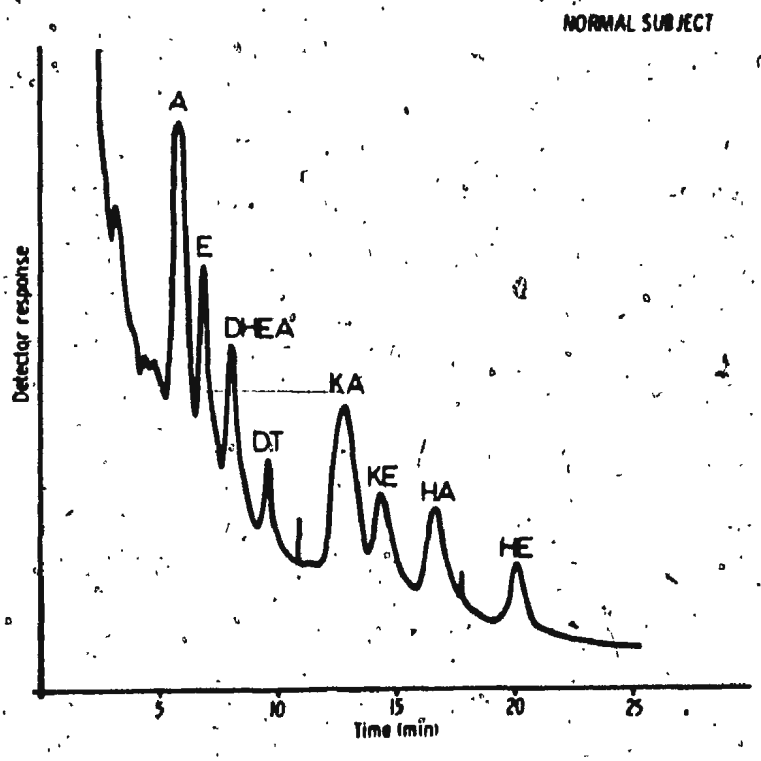
The excretion of these three compounds was again increased in the hypertensive group, but the differences were not statistically significant.

Summation

Table 26 compares the summation of several steroids in the $C_{19}O_2$ and in the $C_{19}O_3$ groups. In the $C_{19}O_2$ group, the total excretion of A+E+DHEA was again markedly reduced in the hypertensive subjects ($P<0.01$), and confirmed the finding in the previous study.

In the $C_{19}O_3$ group, the excretion of the 5α- reduced compounds, KA+HA, was highly increased in the hypertensive subjects ($P<0.01$). The excretion of 5β- reduced compounds, KE+HE, was only slightly increased in the hypertensive group but the difference was not statistically significant. However, the total excretion of KA+HA+KE+HE was significantly increased ($P<0.02$) in the hypertensive subjects reflecting the markedly elevated excretion of 5α- reduced compounds in the same group.

There was no significant difference in the excretion of total 17-ketosteroids, as measured colorimetrically, in the two groups of subjects (Table 26). In addition, the colorimetric values did not correlate well with the $C_{19}O_2$ and $C_{19}O_3$-KS as measured by GLC (Table 25).



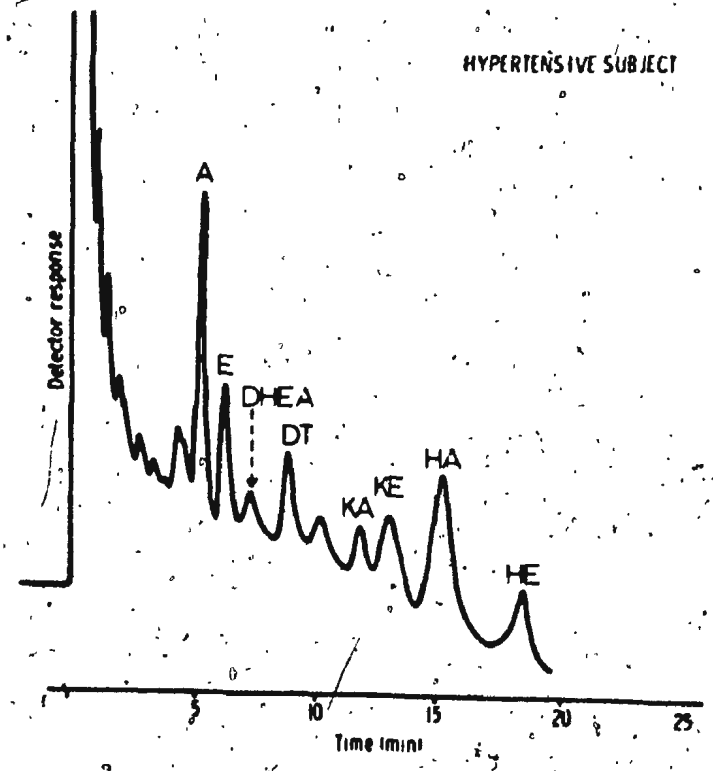


FIG. 12 GLC resolution of the 11-deoxy- and 11-oxy-17-ketosteroids isolated from the urine of NORMAL and HYPERTENSIVE subjects

Table 23

Excretion of individual 11-deoxy- and 11-oxy-17-ketosteroids* (mg/24hr) in the urine of NORMAL subjects

Code	$C_{19}O_2$-KS					$C_{19}O_3$-KS							Total**
	A	E	DHEA	A+E	A+E DHEA	KA	KE	HA	HE	KA+HA	KE+HE	KA+KE HA+HE	17-KS
Males													
M1	1.20	0.54	4.00	1.77	5.77	1.10	1.20	0.71	0.68	1.81	1.85	3.65	13.7
M2	1.11	2.50	1.00	3.61	4.61	0.35	0.22	0.13	0.49	0.48	0.71	1.19	13.6
M3	3.00	0.87	0.42	3.44	4.29	0.09	0.74	0.20	0.31	0.29	1.05	1.34	8.5
M4	1.04	2.40	3.16	2.12	6.60	1.60	1.69	3.27	1.89	4.87	3.58	8.45	10.0
M5	1.54	0.58	6.88	2.23	9.00	1.82	0.66	1.17	0.33	2.99	0.99	3.98	2.7
M6	1.62	1.17	1.35	2.79	4.14	1.10	0.20	1.30	0.22	2.40	0.42	2.82	8.9
M7	2.22	3.06	2.68	5.24	7.96	0.50	1.07	1.20	0.76	1.70	1.83	3.53	5.3
Females													
F1	1.30	1.80	1.40	3.10	4.50	0.50	0.20	0.40	0.50	0.90	0.70	1.60	3.9
F2	2.00	2.60	1.00	4.60	5.60	0.27	0.50	0.05	0.25	0.32	0.75	1.07	3.6
F3	2.70	2.80	1.60	5.50	7.10	0.62	0.38	0.25	0.78	0.87	1.16	2.03	4.1
F4	3.20	1.77	0.50	4.97	5.47	0.53	0.30	0.39	0.71	0.92	1.01	1.93	9.4
F5	0.95	1.08	7.10	2.08	9.13	0.76	0.50	0.38	0.30	1.14	0.80	1.94	5.0
F6	3.30	1.48	1.14	4.78	5.92	0.30	0.52	0.80	0.42	1.10	0.94	2.04	9.7
F7	2.70	0.60	2.51	3.30	5.81	0.24	0.53	0.71	0.30	0.95	0.83	1.78	3.9
F8	1.64	3.61	1.36	5.25	6.61	0.20	1.69	4.60	2.50	4.80	4.19	8.99	6.2
B34	0.99	1.29	0.45	2.28	2.73	0.45	0.55	0.86	0.51	1.31	1.06	2.37	3.0

*Glucuronides plus sulfates

**Colorimetric assay

Table 24

Excretion of individual 11-deoxy- and 11-oxy-17-ketosteroids* (mg/24hr) in the urine of HYPERTENSIVE subjects

Code	$C_{19}O_2$-KS					$C_{19}O_3$-KS							
	A	E	DHEA	A+E	A+E / DHEA	KA	KE	HA	HE	KA+HA	KE+HE	KA+KE / HA+HE	Total** 17-KS
Males													
F65	2.30	1.40	0.40	2.70	3.10	1.20	0.14	1.20	0.97	2.40	1.11	3.51	13.5
F60	1.76	0.49	0.80	2.25	3.05	3.70	2.06	2.16	1.19	5.86	3.25	9.11	4.2
F66	1.65	0.89	0.93	2.50	3.43	1.00	0.81	5.00	1.20	6.00	2.01	8.01	2.9
V44	1.50	0.79	1.00	2.29	3.29	2.00	0.43	1.80	0.15	3.80	0.58	4.38	3.1
Females													
R54	2.20	0.98	0.24	3.18	3.42	2.00	0.70	1.40	0.57	3.40	1.27	4.67	3.2
V32	1.33	1.70	0.20	3.03	3.23	0.96	0.24	0.47	0.16	1.43	0.40	1.83	1.9
V42	4.10	0.35	0.44	4.45	4.89	1.75	0.17	1.03	0.42	2.78	0.59	3.37	2.2
B12	1.70	1.00	0.05	2.70	2.75	0.44	0.41	1.04	0.13	1.48	0.54	2.02	2.3
B64	2.70	0.50	0.05	3.20	3.25	2.20	0.92	2.64	0.51	4.84	1.43	6.27	5.1
F64	3.30	0.51	1.70	3.81	5.51	2.60	1.46	2.30	2.60	4.90	4.06	8.96	2.6
F69	1.54	0.78	0.35	2.32	2.67	0.91	0.70	1.46	0.69	2.37	1.39	3.76	12.1
F62	4.90	2.90	0.88	7.80	8.68	0.70	0.13	1.00	1.60	1.70	1.73	3.43	8.6
F61	1.26	3.00	1.10	4.26	5.36	4.70	2.40	0.68	2.50	5.38	4.90	10.28	2.4
F63	1.12	1.27	0.60	2.39	2.99	0.78	0.58	3.50	0.78	4.28	1.36	5.64	5.5
B59	0.37	0.86	0.75	1.23	1.98	1.00	0.40	0.57	0.24	1.57	0.64	2.21	3.2
V59	4.10	2.70	1.15	6.80	7.95	0.55	1.80	4.20	1.80	4.75	3.60	8.35	2.9
R72	1.48	0.84	0.48	2.32	2.80	0.91	0.98	0.98	0.54	1.89	1.52	3.41	2.1

*Glucuronides plus sulfates

**Colorimetric assay

Table 25

Differences in the mean excretion of individual 11-deoxy- and 11-oxy-17-ketosteroids (mg/24hr) in the urine of NORMAL and HYPERTENSIVE subjects

Steroids		Total Population				Males				Female			
		Mean	±SD	±SE	P value	Mean	±SD	±SE	P value	Mean	±SD	±SE	P value
A	N	1.91	0.81	0.21		1.67	0.65	0.26		2.09	0.87	0.31	
	H	2.20	1.20	0.30	N.S.	1.80	0.30	0.17	N.S.	2.31	1.33	0.38	N.S.
E	N	1.76	0.93	0.21		1.59	0.96	0.39		1.89	0.89	0.31	
	H	1.23	0.83	0.20	< 0.05	0.63	0.19	0.11	< 0.05	1.34	0.90	0.26	< 0.05
DHEA	N	2.22	2.06	0.53		2.78	2.04	0.83		1.89	1.93	0.68	
	H	0.65	0.43	0.10	< 0.01	0.78	0.23	0,13	< 0.02	0.61	0.47	0.13	< 0.01
KA	N	0.65	0.49	0.12		0.94	0.60	0.24		0.43	0.18	0.06	
	H	1.61	1.13	0.28	< 0.01	1.97	0.16	0.61	< 0.05	1.50	1.13	0.32	< 0.01
KE	N	0.68	0.47	0.12		0.82	0.50	0.20		0.57	0.41	0.14	
	H	0.84	0.67	0.17	N.S.	0.86	0.73	0.42	N.S.	0.84	0.65	0.19	N.S.
HA	N	1.03	1.18	0.30		1.14	0.97	0.40		0.94	1.32	0.46	
	H	1.85	1.27	0.32	N.S.	2.54	1.46	0.84	N.S.	1.64	1.13	0.32	N.S.
HE	N	0.68	0.61	0.15		0.67	0.53	0.21		0.69	0.66	0.23	
	H	0.94	0.76	0.19	N.S.	0.88	0.43	0.24	N.S.	0.96	0.83	0.24	N.S.

*Glucuronides plus sulfates

Table 26

Summation of the mean excretion of individual 11-deoxy- and 11-oxy-17-ketosteroids* (mg/24hr) in the urine of NORMAL and HYPERTENSIVE subjects

		Total Population				Males				Females			
Steroids		Mean	±SD	±SE	P value	Mean	±SD	±SE	P value	Mean	±SD	±SE	P value
A+E	N	3.56	1.27	0.33		3.03	1.10	0.45		3.98	1.24	0.44	
	H	3.36	1.64	0.41	N.S.	2.23	0.18	0.10	N.S.	3.65	1.77	0.51	N.S.
A+E+ DHEA	N	4.95	1.70	0.44		6.05	1.75	0.71		5.87	1.65	0.58	
	H	4.02	1.82	0.45	< 0.01	3.72	0.78	0.45	< 0.02	4.27	2.20	0.63	< 0.05
KA+HA	N	1.68	1.38	0.36		1.93	1.51	0.62		1.37	1.24	0.44	
	H	3.46	1.58	0.39	< 0.01	4.51	1.50	0.86	< 0.05	3.14	1.45	0.42	< 0.01
KE+HE	N	1.37	1.02	0.26		1.49	0.98	0.40		1.27	1.04	0.37	
	H	1.79	1.31	0.33	N.S.	1.74	1.01	0.58	N.S.	1.80	1.39	0.40	N.S.
KA+HA+ KE+HE	N	3.04	2.30	0.59		3.57	2.24	0.91		3.64	2.27	0.80	
	H	5.25	2.66	0.66	< 0.02	6.25	2.36	1.36	N.S.	4.94	2.67	0.77	< 0.05
Total** 17-KS	N	6.97	3.51	0.91		5.08	8.96	7.00		3.04	5.42	9.00	
	H	4.58	3.41	0.85	N.S.	4.04	5.93	4.00	N.S.	2.51	4.16	13.00	N.S.

*Glucuronides plus sulfates

**Colorimetric assay

Steroid ratios

The ratio of 5α/5β-reduced compounds, excreted by normal and hypertensive subjects is given in Table 27. The ratio of A/E was significantly increased in the hypertensive group (P 0.05), presumably due to decreased excretion of 5β-reduced compound (E), and confirmed the finding in the previous study.

The ratios of KA/KE, HA/HE, and KA+HE/KE+HE were also elevated markedly (P 0.05, 0.05 and 0.01 respectively) in the hypertensive group, and reflected the increased excretion of 5α-reduced compounds (KA and HA). This suggested that in the formation of $C_{19}O_3$-17-ketosteroids by the hypertensive group, the 5α-reductase activity may be increased relative to the 5β.

Table 28 gives the level of significance of the differences in the excretion of $C_{19}O_2$- and $C_{19}O_3$-KS expressed as mg/24hr urine. The ratios in mg/g creatinine were not calculated since they would be independent of the creatinine concentration.

(iv) Relative 5α- and 5β-reductase activity

The relative proportion of 5α- and 5β-reduced $C_{19}O_2$ and $C_{19}O_3$ steroids were compared between subjects with normal blood pressures and between the hypertensives. Table 29 gives the mean excretion of the individual 5α- and 5β-reduced compounds and the statistical significance of the differences in their excretion.

Normal subjects

The mean excretion of the 5α- and 5β-reduced compounds in the $C_{19}O_2$ and $C_{19}O_3$ groups were similar among the normal subjects. However, there was a tendency for the 5α-compounds, A,KA and KA plus HA to be increased, but the differences were not significant.

Table 27

Ratios of 5α/5β- reduced compounds in the urine of NORMAL and HYPERTENSIVE subjects

Steroid Ratios		Total Population				Males				Females			
		Mean	±SD	±SE	P value	Mean	±SD	±SE	P value	Mean	±SD	±SE	P value
$\frac{A}{E}$	N	1.45	1.20	0.42		1.61	1.09	0.45		1.52	1.26	0.30	
	H	2.84	3.09	0.89	< 0.05	3.29	1.57	0.91	< 0.05	2.95	2.82	0.70	< 0.05
$\frac{KA}{KE}$	N	1.10	0.74	0.26		1.75	1.72	0.70		1.38	1.31	0.34	
	H	2.77	2.53	0.73	< 0.05	4.06	2.91	1.68	N.S.	3.08	2.68	0.67	< 0.05
$\frac{HA}{HE}$	N	1.21	0.73	0.26		2.10	1.83	0.75		1.60	0.40	0.36	
	H	2.76	2.01	0.58	< 0.05	4.80	4.30	2.48	N.S.	3.24	2.86	0.72	< 0.05
$\frac{KA+HA}{KE+HE}$	N	1.05	0.29	0.10		1.84	1.77	0.72		1.39	1.26	0.32	
	H	2.32	1.12	0.32	< 0.01	3.37	1.88	1.09	N.S.	2.57	1.41	0.35	< 0.02

Table 28

Significance of differences* in the excretion of 11-deoxy- and 11-oxy-17-ketosteroids** expressed as mg/g creatinine*** as compared to mg/24hr**** in the urine of NORMAL and HYPERTENSIVE subjects

Steroids	Total Population		Males		Females	
	mg/g***	mg/24hr****	mg/g***	mg/24hr****	mg/g***	mg/24hr****
Individual values						
A	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
E	0.05	0.05	N.S.	0.05	0.05	0.05
DHEA	0.02	0.01	0.05	0.02	0.05	0.02
KA	0.01	0.01	0.01	0.05	0.01	0.01
KE	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
HA	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
HE	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Summations						
A+E	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
A+E+DHEA	0.02	0.01	N.S.	0.02	0.02	0.05
KA+HA	0.01	0.01	0.05	0.05	0.05	0.01
KE+HE	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
KA+HE+KE+HE	0.05	0.02	N.S.	N.S.	N.S.	0.05
Steroid ratios						
A/E		0.05		0.05		0.05
KA/KE		0.05		N.S.		0.05
HA/HE		0.05		N.S.		0.05
KA+HE/KE+HE		0.01		N.S.		0.02
Total 17-KS***		N.S.		N.S.		N.S.

*Student 't' test; **Glucuronides plus sulfates; ***Colorimetric assay; P

Hypertensive subjects

Statistical differences were evident in the excretion of the 5α- and 5β- pairs in both the $C_{19}O_2$ and $C_{19}O_3$ groups by the hypertensives. In all cases, the 5α- compounds were excreted in greater amounts than the 5β. Among the $C_{19}O_3$ compounds the possible 5α- directing influence of an 11-oxy-group was evident in the excretion of KA and HA.

Comparison of the levels of 5α- and 5β- reduced compounds excreted by the normal and hypertensive subjects indicates a relative deficiency of 5β- reductase activity in the formation of the $C_{19}O_2$-17-keto-steroids in hypertension. By contrast, the 5α- reductase activity appears to be significantly increased in the reduction of the 11-oxy-17-ketosteroids by the hypertensive group.

(v) Effect of age on the excretion of 11-oxygenated 17-ketosteroids

The effect of age on the excretion of the $C_{19}O_3$-KS in the urine of normal and hypertensive subjects is illustrated in Fig. 13. The majority of the hypertensive subjects exhibited higher levels of KA and HA than the normal subjects over the whole range of ages studied (21 - 69 years).

(vi) Effect of blood pressure on the excretion of 11-oxygenated 17-ketosteroids

The effect of increasing diastolic and systolic blood pressure on the excretion of $C_{19}O_3$-KS is shown in Figs. 14 and 15 respectively. No definite correlation between these parameters were observed except that the excretion of KA and HA appeared to increase with diastolic pressure over 90 and systolic pressure over 140. However, several individuals were atypical.

Table 29

Differences in the mean excretion of 5α- and 5β- reduced 17-ketosteroids (mg/24hr) in the urine of NORMAL and of HYPERTENSIVE subjects

Normal subjects

	Total Population				Males				Females			
Steroids	Mean	±SD	±SE	P value	Mean	±SD	±SE	P value	Mean	±SD	±SE	P value
A	1.91	0.80	0.21		1.67	0.65	0.26		2.09	0.82	0.31	
E	1.76	0.96	0.25	N.S.	1.59	0.96	0.39	N.S.	1.89	0.92	0.34	N.S.
KA	0.65	0.50	0.13		0.94	0.60	0.24		0.43	0.19	0.07	
KE	0.68	0.47	0.12	N.S.	0.82	0.50	0.20	N.S.	0.57	0.43	0.14	N.S.
HA	1.03	1.18	0.30		1.14	0.97	0.39		0.94	1.32	0.46	
HE	0.68	0.62	0.16	N.S.	0.67	0.53	0.21	N.S.	0.69	0.66	0.26	N.S.
KA+HA	1.68	1.38	0.36		2.08	1.45	0.59		1.39	1.24	0.44	
KE+HE	1.37	1.02	0.26	N.S.	1.49	0.98	0.40	N.S.	1.27	1.04	0.37	N.S.

Hypertensive subjects

A	2.20	1.19	0.29		1.80	0.30	0.17		2.31	1.33	0.37	
E	1.23	0.80	0.19	< 0.02	0.89	0.33	0.18	< 0.01	1.34	0.88	0.24	< 0.05
KA	1.61	1.13	0.27		1.97	1.06	0.61		1.50	1.13	0.31	
KE	0.84	0.67	0.16	< 0.05	0.86	0.73	0.42	< 0.05	0.84	0.63	0.17	< 0.05
HA	1.85	1.27	0.30		2.54	1.46	0.84		1.64	1.13	0.30	
HE	0.94	0.74	0.18	< 0.05	0.88	0.43	0.24	< 0.05	0.96	0.83	0.24	< 0.05
KA+HA	3.46	1.60	0.39		4.51	1.50	0.86		3.14	1.46	0.40	
KE+HE	1.79	1.31	0.32	< 0.01	1.74	1.01	0.58	< 0.02	1.80	1.38	0.40	< 0.05

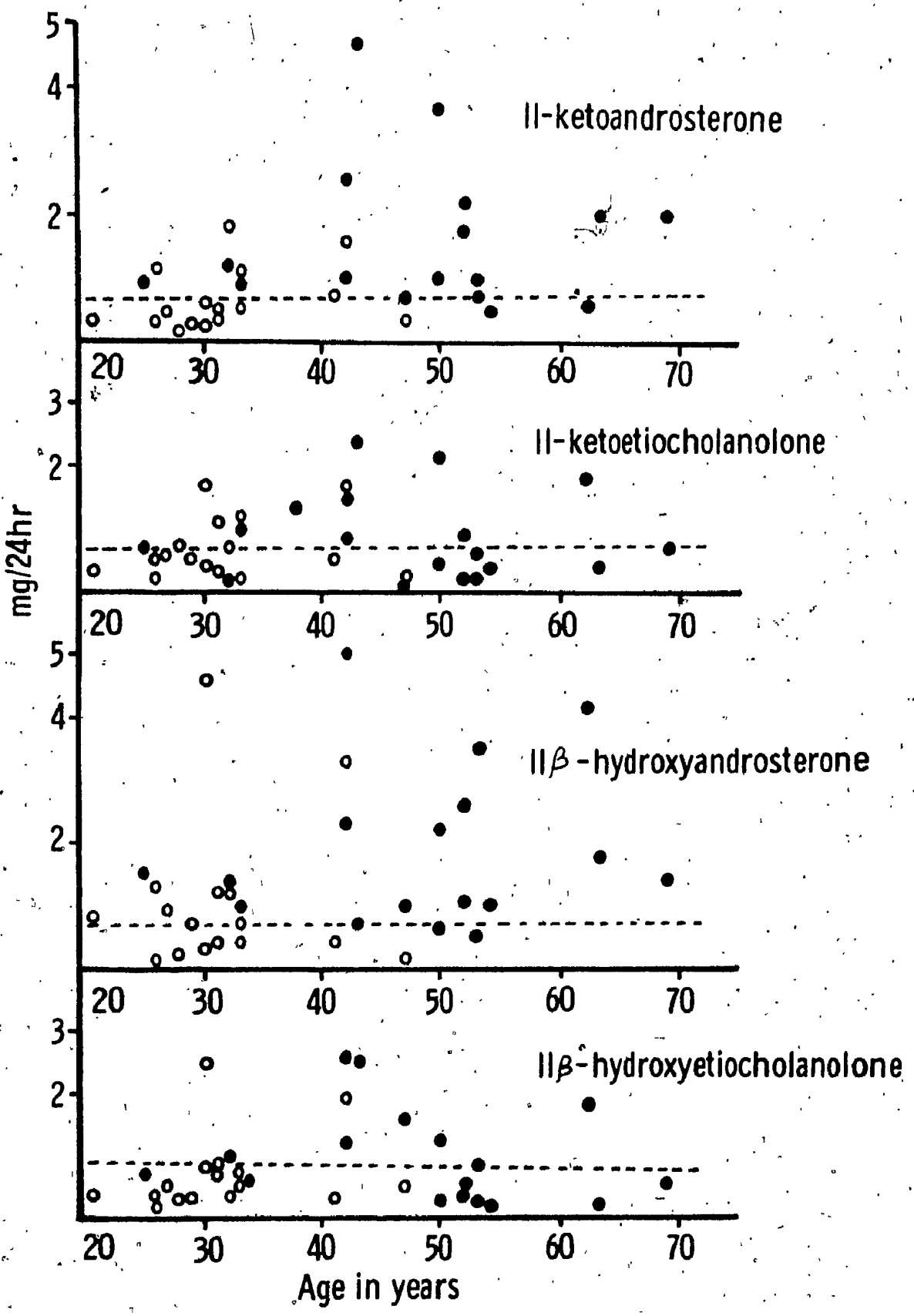


Fig. 13 Excretion of 11-oxy-17-ketosteroids in the urine of NORMAL and HYPERTENSIVE subjects with increasing age

Normal subjects ○ Hypertensive subjects ●

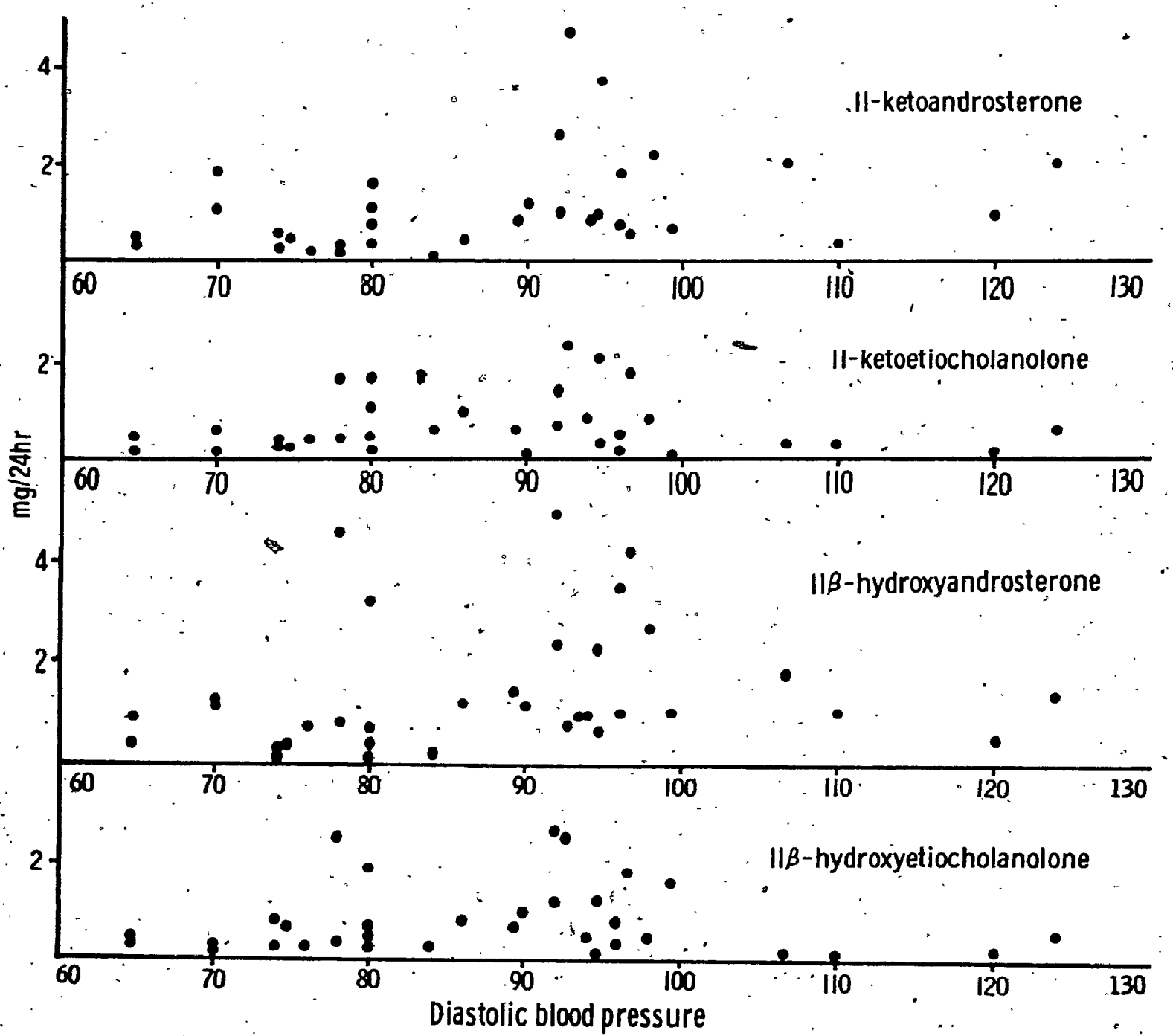


Fig. 14 Excretion of 11-oxy-17-ketosteroids in the urine of NORMAL and HYPERTENSIVE subjects with increasing diastolic blood pressure

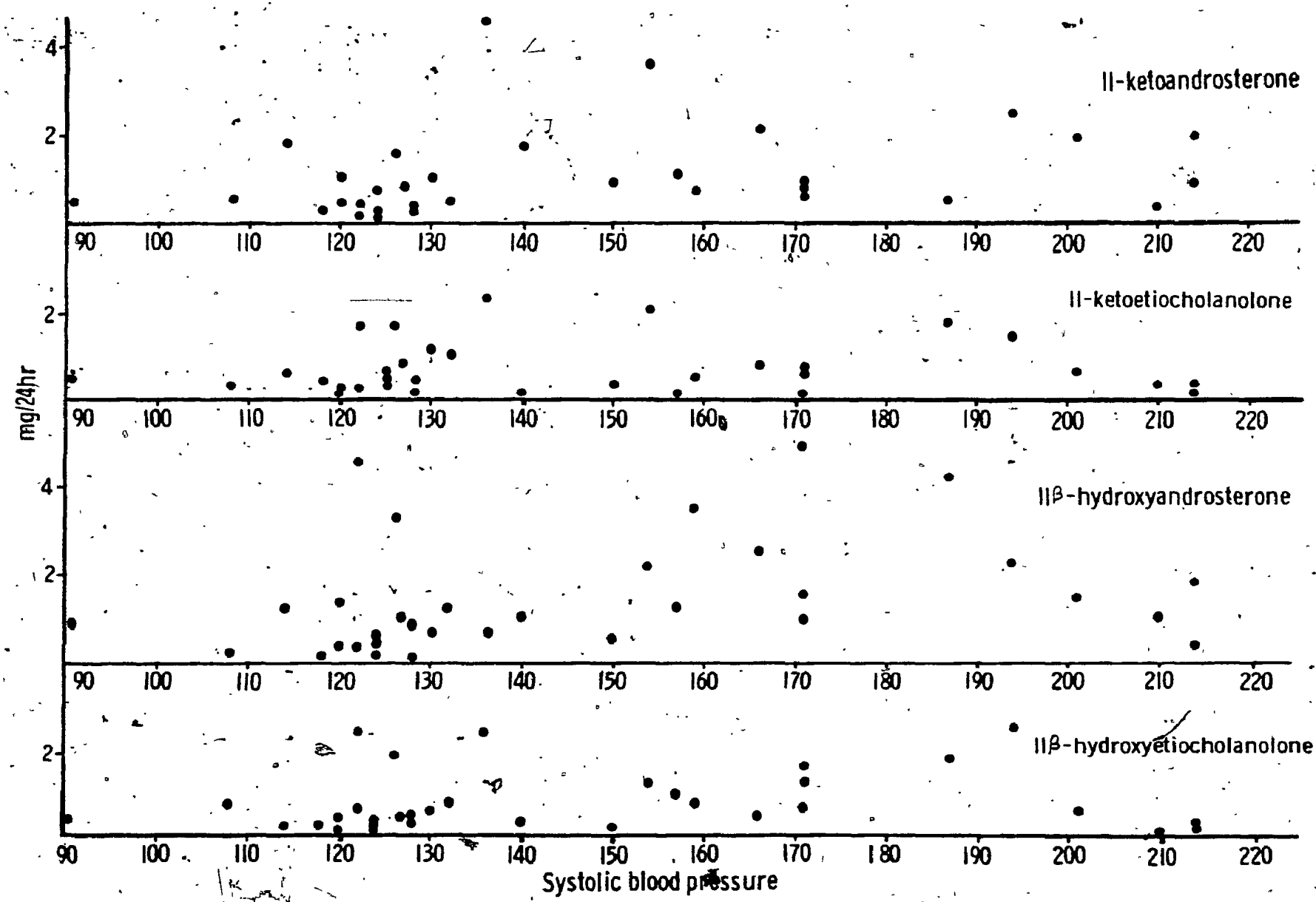


Fig. 15 Excretion of 11-oxy-17-ketosteroids in the urine of NORMAL and HYPERTENSIVE subjects with increasing systolic blood pressure

(vii) Possible diagnostic value of DHEA and the ratio A+E/DHEA in essential hypertension

The excretion of DHEA and the ratio of A+E/DHEA in the normal and hypertensive subjects are illustrated in Fig. 16. It was of interest to note that 82% of the normal subjects excreted more than 1.2mg DHEA/24hr urine, whereas 90% of the hypertensive subjects excreted less than 1.2mg/24hr.

For the ratio A+E/DHEA, it was found that 85% of the normal subjects had ratios less than 4, whereas 73% of the hypertensive subjects had ratios greater than 4.

It would seem, therefore, that there is a good correlation between the excretion of DHEA or the ratio of A+E/DHEA in the essential hypertensive population studied, which could be of value in supplementing the data from blood pressure measurements. Whether it has any further value in differentiating essential from other forms of hypertension, has not been determined.

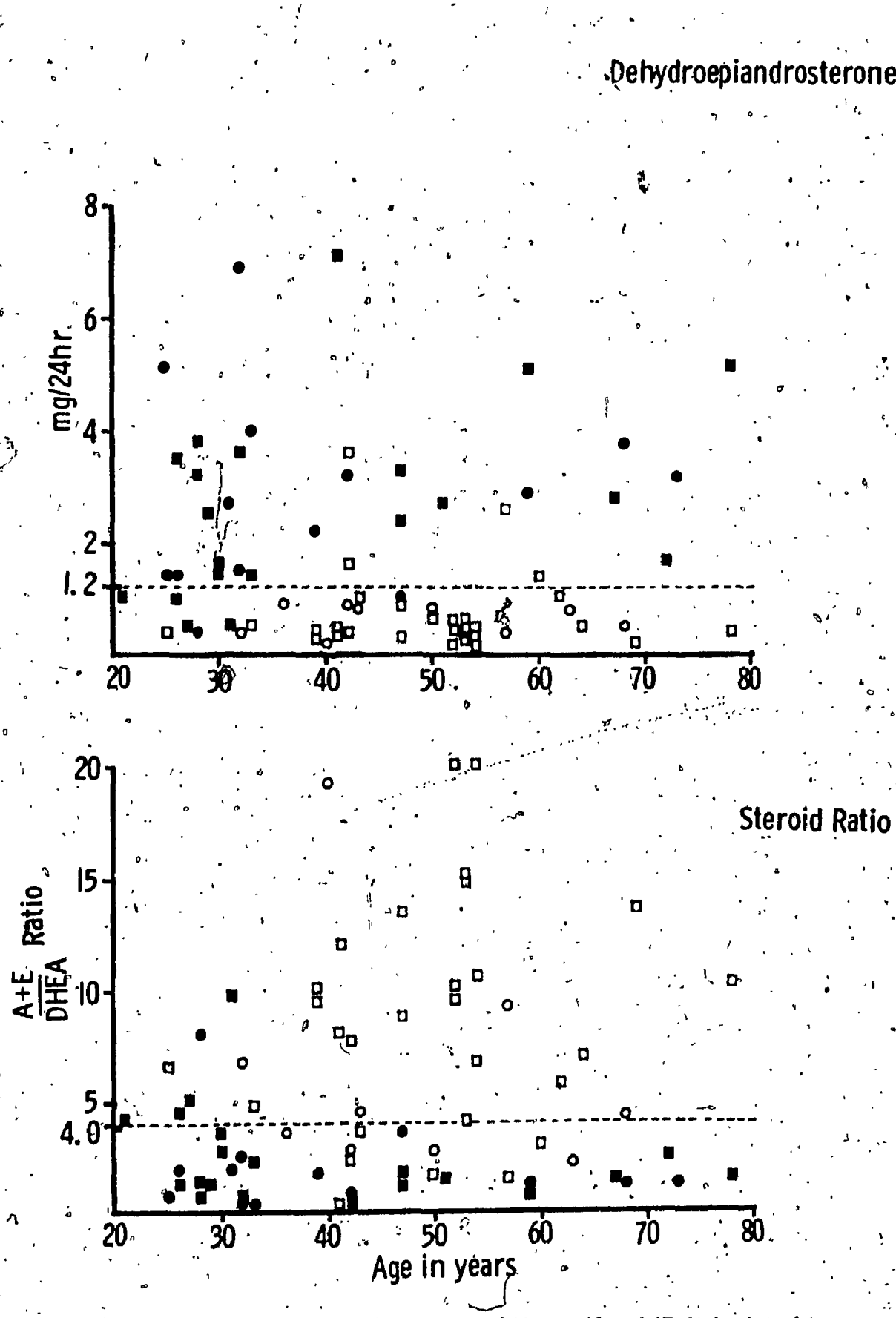


Fig. 16 Excretion of dehydroandrosterone and the ratio of 17-ketosteroids excreted by NORMAL and HYPERTENSIVE subjects of different ages

Hypertensive subjects Normal subjects

Females □ ■

Males ○ ●

SECTION IV

Studies on the 17-ketogenic steroids excreted in the urine of NORMAL and HYPERTENSIVE subjects

(i) GLC resolution

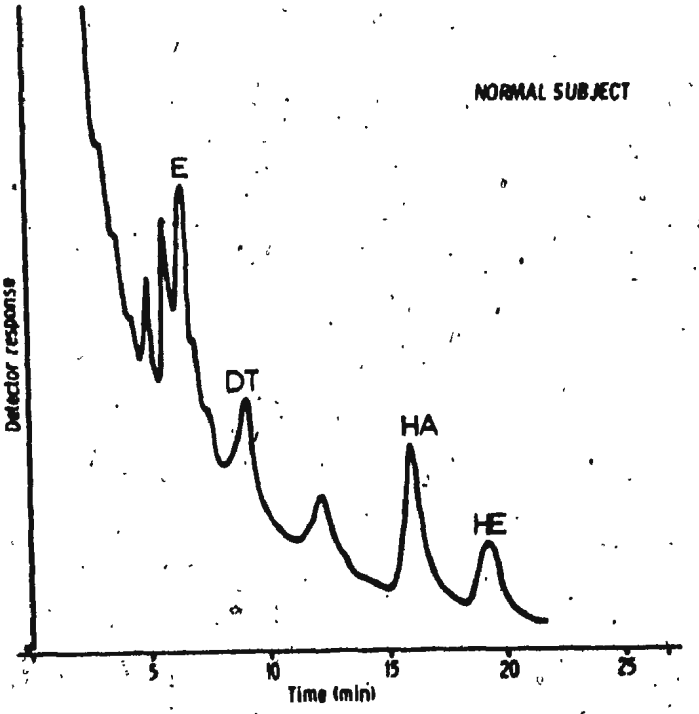
The C_{21}-corticosteroid fraction which was isolated from the urine of normal and hypertensive subjects was measured indirectly as 17-ketogenic steroids by oxidation to 17-ketosteroids as described in the methodology section. Each component of the 17-ketogenic steroid fraction was then resolved by GLC as its TMSE derivatives. Fig. 17 compares the resolution of etiocholanolone, 11β-hydroxyandrosterone and 11β-hydroxy etiocholanolone, which were derived from the corticosteroid fractions in the urines of a normal and a hypertensive subject. The possible source of these steroids in the 17-ketogenic steroid fraction has been discussed in the methodology section. The spectra illustrate a satisfactory resolution of the steroids studied and the absence of interfering peaks.

(ii) Excretion of individual 17-Ketosteroids derived from the 17-Ketogenic steroids

Table 30 gives the individual values for the excretion of the three major 17-ketosteroids in the urine of male and female normal and hypertensive subjects. The individual glucuronide and sulfate conjugates were not analysed in this study.

(iii) Comparison of differences in the excretion of 17-Ketosteroids and 11-deoxy-17-ketosteroids derived from the 17-ketogenic steroids (Tables 31 and 32

Etiocholanolone was found to be a major steroid metabolite that was derived from the 11-deoxy-corticosteroids by the ketogenic reaction.



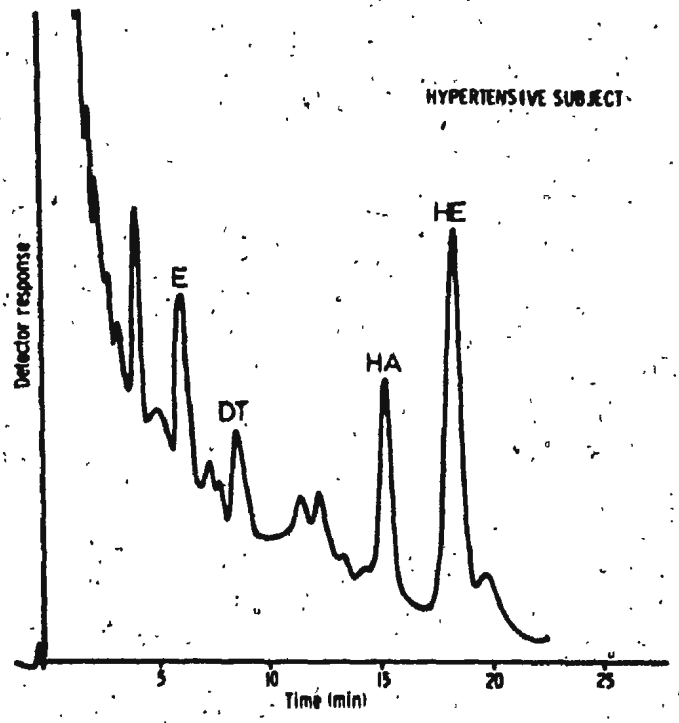


FIG. 17 GLC resolution of the 17-ketogenicsteroids derived from the corticosteroid fraction isolated from the urine of NORMAL and HYPERTENSIVE Subjects

Table 30

Excretion of individual 17-ketosteroids* (mg/24hr) derived from the 17-ketogenic steroids excreted in the urine of NORMAL and HYPERTENSIVE subjects

Normal								Hypertensive							
Code	E	HA	HE	HA+HE	E HA +HE	Total KGS**	Total OHCS (P-S)	Code	E	HA	HE	HA+HE	E+HA +HE	Total KGS**	Total OHCS (P-S)
Males								**Males**							
M1	0.41	1.67	1.98	3.65	4.06	10.6	5.7	F60	3.80	2.50	2.40	4.90	8.70	4.2	1.6
M2	1.10	0.98	1.98	2.96	4.06	11.5	6.2	F65	1.11	6.31	7.07	13.38	14.49	8.0	5.7
M3	1.20	1.68	2.14	3.82	5.02	6.8	4.4	F66	1.80	4.60	4.90	9.50	11.30	7.5	4.2
M4	1.59	1.54	4.43	5.97	7.56	8.5	5.6	V44	0.70	0.83	0.94	1.77	2.47	0.5	0.4
M5	1.20	1.70	1.90	3.60	4.80	7.0	2.9								
M6	1.30	2.03	2.16	4.19	5.49	27.6	19.0								
M7	1.04	0.88	2.44	3.32	4.36	7.7	9.5								
Females								**Females**							
F1	0.65	1.90	1.60	3.50	4.15	6.5	4.4	B12	1.08	5.20	3.90	9.10	10.18	2.2	1.4
F2	0.58	2.22	2.79	5.01	5.59	11.4	3.4	B59	0.59	3.80	3.80	6.60	7.55	1.8	1.4
F3	0.74	0.86	1.65	2.51	3.25	6.7	1.9	B64	0.71	1.01	2.89	3.90	4.61	3.4	2.7
F4	0.10	0.64	0.61	1.25	1.35	6.4	2.1	F61	2.00	2.50	5.90	8.40	10.40	0.7	0.7
F5	1.00	1.49	1.64	3.13	4.13	10.9	2.7	F62	1.01	6.14	6.42	12.56	13.84	6.6	4.5
F6	0.58	1.73	1.36	3.09	3.67	10.5	4.2	F63	1.70	3.50	6.50	10.00	11.70	3.6	2.6
F7	1.90	2.70	2.80	5.50	7.40	3.8	1.9	F64	1.70	4.70	4.70	9.40	11.10	3.6	2.5
F8	1.07	2.70	5.20	7.90	8.97	7.7	2.1	F69	0.54	1.13	1.47	2.60	3.14	-	3.0
B34	2.58	3.30	4.70	8.00	10.58	6.1	1.8	R72	1.14	2.32	1.73	4.05	5.19	5.5	1.0
								V32	0.65	2.60	2.20	4.80	5.45	4.4	3.3
								V42	2.14	3.20	4.78	7.98	10.12	3.6	1.4
								V59	4.80	0.69	0.95	1.64	6.44	5.9	4.9
								R54	0.85	4.89	6.90	11.79	12.64	15.2	6.5

*Glucuronides plus sulfates; **Colorimetric assay; (P-S) Porter Silber Chromogen

Table 31

Differences in the excretion of 17-ketosteroids* (mg/24hr) derived from the 17-ketogenic steroids excreted in the urine of NORMAL and of HYPERTENSIVE subjects

		Total Population				Males				Females			
Steroids		Mean	±SD	±SE	P value	Mean	±SD	±SE	P value	Mean	±SD	±SE	P value
Individual values													
E	N	1.06	0.58	0.15		1.12	0.33	0.13		1.02	0.72	0.25	
	H	1.57	1.11	0.28	N.S.	1.85	1.19	0.69	N.S.	1.48	1.08	0.31	N.S.
HA	N	1.75	1.23	0.32		1.50	0.83	0.34		1.95	1.47	0.51	
	H	3.22	2.07	0.52	0.02	3.56	2.35	1.35	N.S.	3.13	1.97	0.57	0.05
HE	N	2.46	0.71	0.18		2.43	0.38	0.15		2.48	0.83	0.29	
	H	3.97	1.76	0.44	0.01	3.83	2.07	1.20	N.S.	4.01	1.64	0.47	0.05
Summation													
HA+HE	N	4.21	1.79	0.46		3.93	0.90	0.37		4.43	2.22	0.78	
	H	7.20	3.64	0.91	0.01	7.39	4.42	2.55	N.S.	7.14	3.36	0.97	0.05
E+	N	5.28	2.24	0.58		5.05	1.13	0.46		5.45	2.80	0.99	
HA+HE	H	8.78	3.59	0.90	0.001	9.24	4.46	2.55	N.S.	8.64	3.29	0.95	0.02
Steroid ratios													
HA	N	0.71	0.64	0.17		0.61	0.73	0.30		0.78	0.40	0.14	
HE	H	0.81	0.55	0.12	N.S.	0.92	0.06	0.03	N.S.	0.78	0.60	0.17	N.S.
E	N	0.24	0.09	0.00		0.28	0.07	0.00		0.21	0.09	0.03	
HA+HE	H	0.37	0.65	0.16	N.S.	0.36	0.26	0.15	N.S.	0.38	0.73	0.21	N.S.
Total**	N	9.36	5.19	1.34		11.38	6.82	3.78		7.78	2.44	0.86	
17-KGS	H	4.72	3.47	0.89	0.01	5.05	3.00	1.23	N.S.	4.62	3.60	1.08	0.05

*Glucuronides plus sulfates; **Colorimetric assay

Table 32

Significance of differences* in the excretion of 17-ketosteroids** derived from the 17-ketogenic steroids and expressed as mg/g creatinine*** as compared to mg/24hr in the urine of NORMAL and HYPERTENSIVE subjects

Steroids	Total Population		Males		Females	
	mg/g Creatinine	mg/24hr Urine	mg/g Creatinine	mg/24hr Urine	mg/g Creatinine	mg/24hr Urine
Individual values						
E	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
HA	0.05	0.02	0.05	N.S.	0.05	0.05
HE	0.02	0.01	0.05	N.S.	0.05	0.05
Summation						
HA+HE	0.02	0.01	N.S.	N.S.	0.05	0.05
E/HA+HE	0.01	0.001	0.01	N.S.	0.05	0.02
Steroid ratios						
HA+HE	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
E/HA+HE	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Total 17-KGS***	N.S.	0.01	N.S.	N.S.	N.S.	0.05

* Student 't' test' P

** Glucuronides plus sulfates

*** Colorimetric assay

No evidence, however, was obtained for the presence of the 5α- reduced steroid, androsterone, in the urine of the normal and hypertensive subjects. The mean excretion of E was slightly increased in the hypertensive group over the normal group, but the difference was not statistically significant.

11-Oxy-17-ketosteroids derived from the 17-ketogenic steroids

The mean excretion of both the 5α- (HA) and 5β- (HE) 11-oxy-17-ketosteroid was higher in the hypertensive group, as compared to the group of normal subjects. When the ratio of HA/HE was compared in the two groups, no significant difference was evident, presumably indicating equal 5α- and 5β-reductase activity in the formation of these metabolites which are derived from the corticosteroids. The summations of HA and HE indicated a greater excretion of these 11-oxy-17-ketosteroids by the hypertensives.

The colorimetric analysis of the 17-ketogenic steroids revealed a decreased excretion of these compounds by the hypertensive group that was statistically significant (P 0.01). However, the summation of the individual major 17-ketogenic steroids, namely E, HA and HE, as measured by GLC, again failed to correlate well with the colorimetric assay, since the former group of compounds was significantly elevated in the hypertensive group (P 0.001).

11-oxygenation index

The ratio of the 11-deoxy-17-ketosteroid, E and the 11-oxy-17-ketosteroids, HA and HE gives an "11-oxygenation index". No significant difference was found in this value when calculated for the normal and hypertensive groups. This may suggest that no significant difference exists in the 11β-dehydrogenase enzyme activity in the metabolism of corticosteroids by the two groups.

(iv) Relative 5α- and 5β- reductase activity

In the previous section the excretion of 5α- and 5β- reduced 17-ketosteroids by the normal and hypertensive subjects were compared.

In Table 33 a comparison is made of the relative 5α- and 5β- reductase activities among the normal subjects, and also among the hypertensive subjects by comparing the levels of 11β-hydroxy androsterone and 11β-hydroxy etiocholanolone obtained from the C_{21}-corticosteroid fraction in each group.

The mean excretion of 11β-hydroxy etiocholanolone was slightly higher than 11β-hydroxy androsterone in both the normal and hypertensive groups, but the differences were not statistically significant. This may suggest that in the metabolism of corticosteroids by both normal and hypertensive subjects, the 5α- and 5β-reductase activities were approximately equal, and may reflect the influence of both the 11-oxy function and the corticosteroid side chain.

(v) Effect of age on the excretion of 17-ketosteroids derived from the 17-ketogenic steroids

Fig. 18 illustrates the effect of advancing age on the excretion of 17-ketosteroids by normal and hypertensive subjects. The regression curve does not show any significant change in the levels of these steroids with increasing age. The majority of the hypertensive subjects excreted higher levels of 11β-hydroxy androsterone and 11β-hydroxy etiocholanolone than the normal subjects over the age range studied.

(vi) Effect of blood pressure on the excretion of 17-ketosteroids derived from the 17-ketogenic steroids

The effect of increasing diastolic and systolic blood pressure on the excretion of 17-ketosteroids is shown in Figs. 19 and 20 respectively. No correlation between these parameters was found.

Table 33

Differences in the mean excretion of 5α- and 5β- reduced 17-ketosteroids* (mg/24hr) derived from the 17-ketogenic steroids excreted in the urine of NORMAL and of HYPERTENSIVE subjects

	Total Population				Males				Females			
Steroids	Mean	±SD	±SE	P value	Mean	±SD	±SE	P value	Mean	±SD	±SE	P value
Normal subjects												
HA	1.75	1.23	0.32		1.50	0.83	0.34		1.95	1.47	0.52	
HE	2.46	0.71	0.18	N.S.	2.43	0.38	0.15	N.S.	2.48	0.83	0.29	N.S.
Hypertensive subjects												
HA	3.23	2.07	0.52		3.56	2.35	1.35		3.13	1.97	0.57	
HE	3.97	1.76	0.44	N.S.	3.83	2.07	1.20	N.S.	4.01	1.64	0.47	N.S.

*Glucuronides plus sulfates

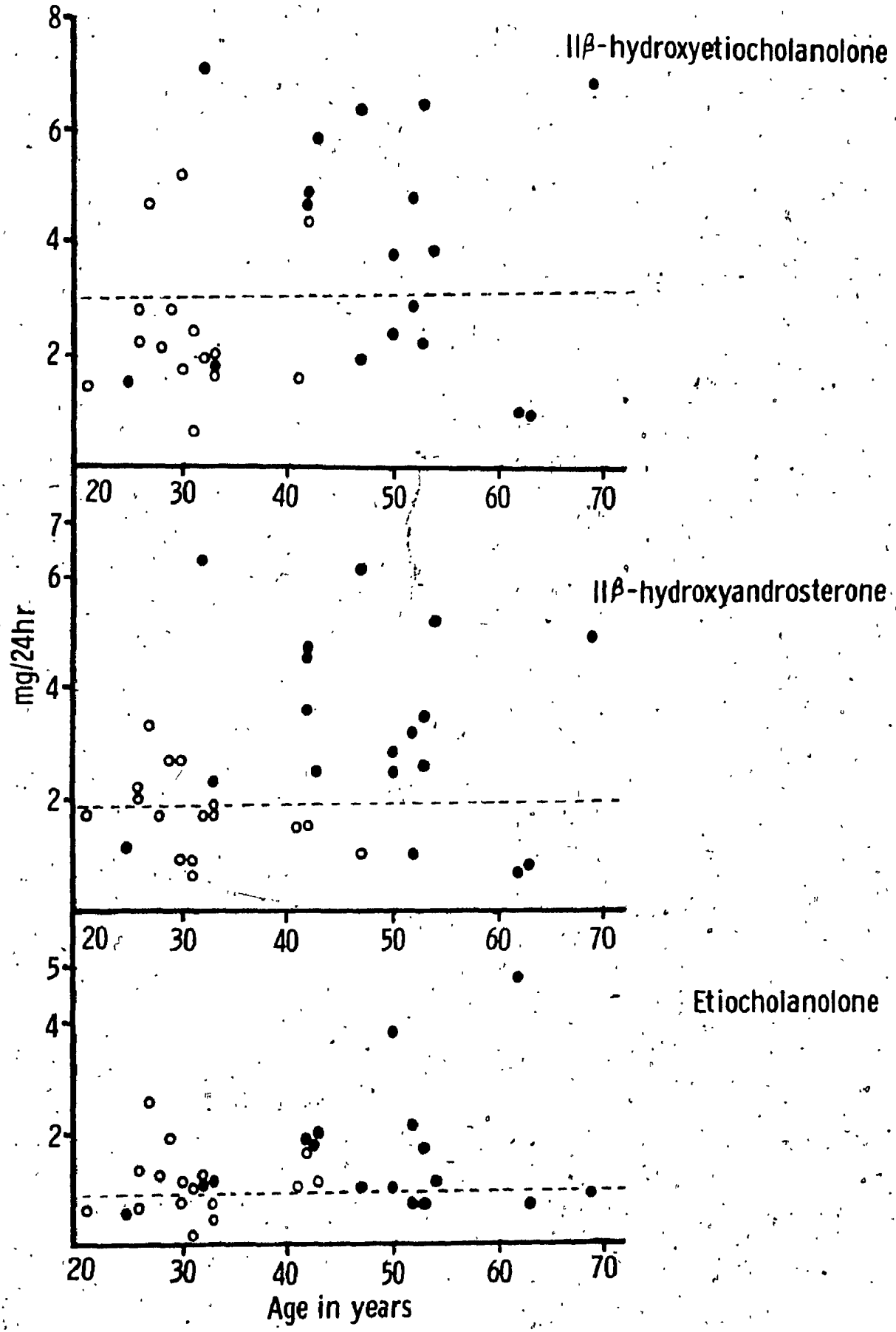


Fig. 18 Excretion of 17-ketogenicsteroids in the urine of NORMAL and HYPERTENSIVE subjects with increasing age

Normal subjects ○ HYPERTENSIVE subjects ●

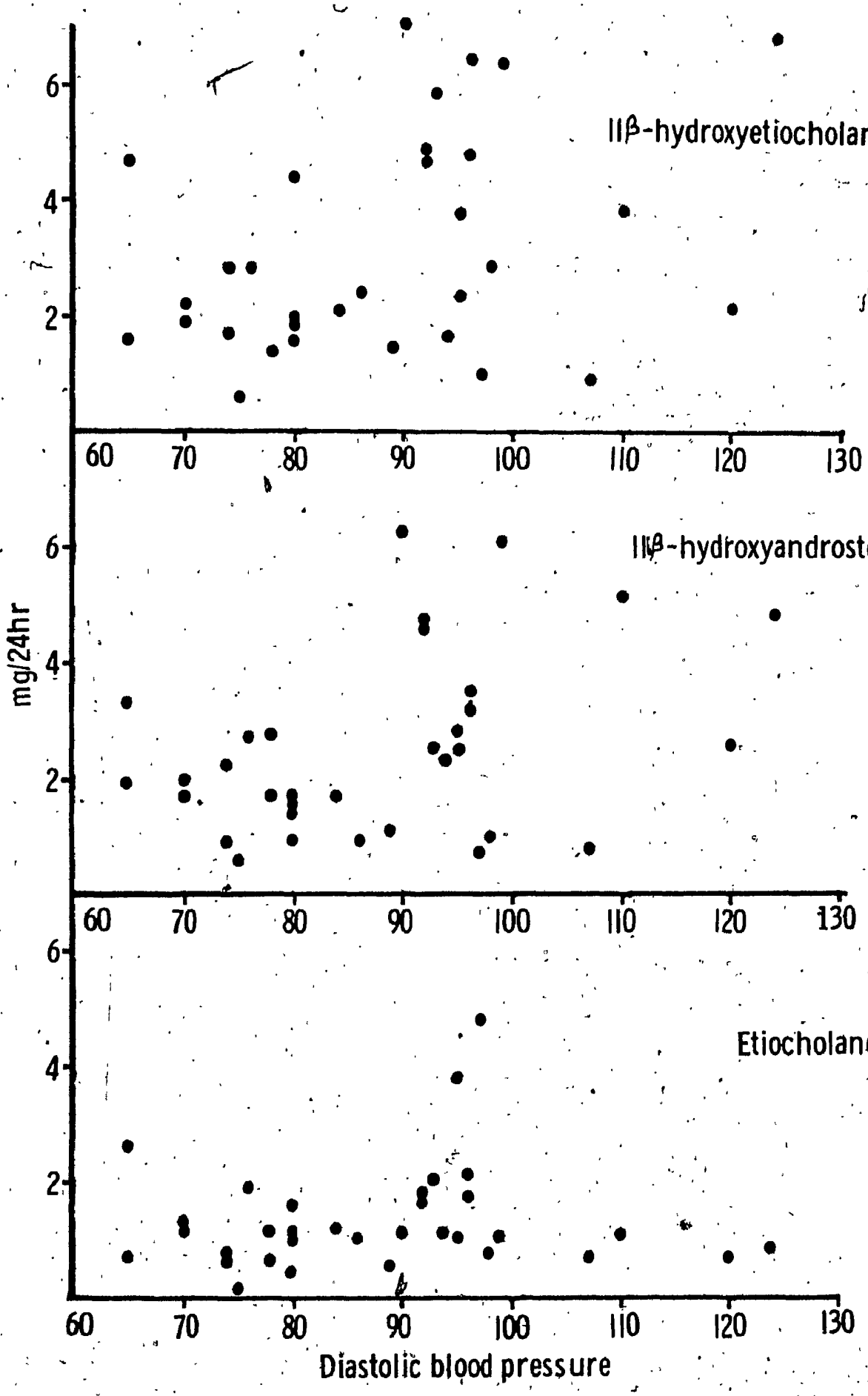


Fig. 19 Excretion of 17-ketogenicsteroids in the urine of NORMAL and HYPERTENSIVE subjects with increasing Diastolic blood pressure

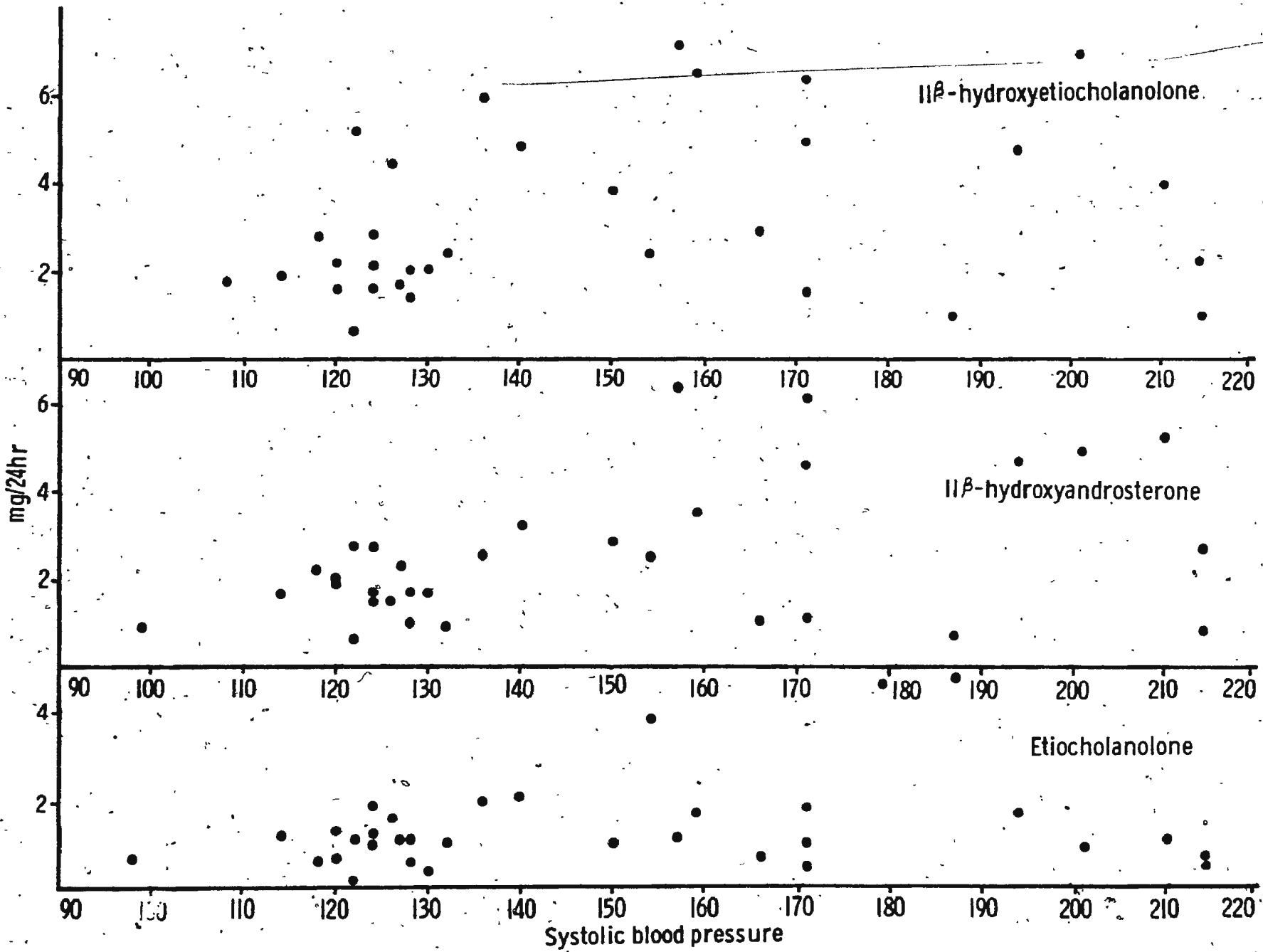


Fig. 20 Excretion of 17-ketogenicsteroids in the urine of NORMAL and HYPERTENSIVE subjects with increasing systolic blood pressure

DISCUSSION

Steroid metabolism has been extensively investigated in the normal healthy human and in a variety of disease states during the past three decades, but relatively little attention has been given to alterations in steroid metabolism that occur in essential hypertension until relatively recently. Several reports have indicated that essential hypertensives may excrete abnormal levels of pregnanediol, pregnanetriol, pregnenetriol (Nowaczynski et al, 1960, 1964; Vermeulen and Ven der Straeten, 1963), DHEA-sulfate (Nowaczynski et al, 1968) and corticosteroid metabolites (Kornel and Motohashi, 1965; Kornel and Takeda, 1967, Kornel et al, 1969). In addition, Edwards has isolated an unusual 11-oxygenated steroid metabolite from the urine of hypertensive children (Edwards et al, 1968).

In the present thesis these studies have been extended by systematically investigating the 11-deoxy and 11-oxygenated 17-ketosteroids, together with the 17-hydroxycorticosteroids that are excreted in the urine of normal and essential hypertensive subjects. The majority of the normal and essential hypertensive subjects resided in four Newfoundland communities and had taken part in a hypertension survey. The epidemiological aspects of this survey have been reported elsewhere (Abbott et al, 1971a,b; Fodor et al, 1973). Single 24 hour urines, collected from both male and female volunteers in each community, and were frozen immediately after collection and kept deep-frozen until analysed. A second group of subjects was drawn from among normal laboratory personnel in good health, and formed part of the normotensive group.

The distribution of ages in both male and female groups is rather broad since the urines were collected from all individuals who were willing to participate in the study. In this respect the selection of subjects was unbiased and included a broad spectrum of individuals. However, the failure to

select subjects at this stage required that the results be examined in several ways as discussed below.

It can be seen from Tables 14 and 15 that there was considerable variability in the body weight, volumes of urine voided and grams of creatinine excreted by individuals. Although it is recognized that the excretion of creatinine is a function of body muscle mass, no significant correlation could be made in the present study. Interestingly, several of the normotensive subjects from among the normal laboratory personnel excreted less than 1gm of creatinine per day. In order to include as many subjects as possible for the steroid studies, it was decided to analyse all the urines collected, irrespective of the age, body weight or creatinine excretion. Steroids were then expressed in two ways; as mg/24hr urine and as mg steroid/gm creatinine. The latter was included in order to overcome variations in the completeness of urine collection which may have occurred. Tables 16, 20, 22, 28 and 32 indicate that the results expressed in this way were not significantly different from results expressed as mg/24hr urine.

Three normotensive and seven hypertensive subjects excreted less than 0.8g creatinine per 24hr, which was more than one standard deviation below the mean as calculated for the population studied. When these subjects were excluded from the calculations, it was observed that the statistically significant differences which had been found in the excretion of several steroids were not altered. It was therefore concluded that the analysis of results obtained from all of the individuals studied was valid and was not biased by including those subjects with low creatinine excretion rates.

Excretion of aldosterone in essential hypertension

The urinary excretion of aldosterone was determined for the subjects in the present study primarily in order to eliminate primary aldosteronism as a cause of the hypertension. There is considerable controversy

as to whether the excretion of aldosterone is altered in essential hypertension, so it was of interest to compare the results with other workers.

The double isotope derivative assay procedure that was developed for the estimation of urinary aldosterone involves several modifications of existing procedures as discussed in the Methods section, and reported by Dey et al, (1972). The overall recovery was rather low (9.3% ± 1.8), but both precision and reproducibility were good. Since the assay includes preliminary hydrolysis with cold acid, it measures both the free and acid labile glucuronide conjugated forms of this steroid. This is the conventional procedure for the estimation of urinary aldosterone.

Thirty-three normal subjects excreted 1.1 - 20.4ug aldosterone per 24 hours as compared to 2.6 - 18.7ug/24hr excreted by thirty-seven hypertensive subjects. The difference between the two groups was therefore not statistically significant. There is an extensive literature on aldosterone excretion in essential hypertension and different ranges have been reported by several workers. Both normal and hypertensive groups have been reported as excreting similar ranges of aldosterone by: Slaton and Biglieri, 1965 (5-25ug/24hr), Ledingham et al, 1967 (2-30ug/24hr), Biglieri et al, 1970 (4-17ug/24hr), 1972 (2-23ug/24hr), Brunner et al, 1972b (1-25ug/24hr). Others have reported elevated values for the hypertensive group: Nowaczynski et al, 1957 (N:2.2-10 and H:1.5-40ug/24hr), Garst et al, 1960 (N:3.1-21.1 and H:3.1-39.1ug/24hr), Genest et al, 1960a (N:1-11 and H:1-36ug/24hr), 1961 (N:1-9 and H:1-36ug/24hr) and Venning et al, 1961 (N:2-12 and H:3.3 - 19.6ug/24hr).

The secretion rate of aldosterone in benign essential hypertension may be either normal (Laragh et al, 1960a,b; 1963; Kaplan and Silah, 1964; Slaton and Biglieri, 1965), or low (Nowaczynski et al, 1971, 1972), although recent evidence suggests that the metabolic clearance rate of aldosterone may be decreased in benign essential hypertension (Nowaczynski et al, 1971, 1972; Lommer et al, 1972). If the latter is a characteristic of essential

hypertension, one would expect the excretion rate of aldosterone to also be reduced, but as indicated above this is not invariably so. An alteration in the hepatic metabolism of aldosterone in essential hypertension has been suggested from observations that the urinary excretion of the "oxo-conjugate" of aldosterone is higher than its tetrahydro glucuronide conjugate. The significance of this finding is not clear but may indicate a possible alteration in the hepatic inactivation of aldosterone in essential hypertension (Nowaczynski et al, 1972).

Excretion of dehydroepiandrosterone

The first report that the excretion of dehydroepiandrosterone was reduced in the urine of hypertensives was based on the failure to detect any DHEA (Kolbel et al, 1964). More precise methods however, have revealed a marked reduction in the excretion of DHEA-sulfate in the urine of essential hypertensives as compared to normal subjects (Nowaczynski et al, 1968). The hypertensive subjects studied in the present work also exhibited a significant reduction in the excretion of DHEA-sulfate with a mean of 0.46mg/24hr as compared to a mean of 3.07mg/24hr (P 0.001) excreted by the normal subjects. By contrast, the excretion of the glucuronide conjugate of DHEA was not altered, which also confirms the work of Nowaczynski et al (1968). The reduced excretion of DHEA-sulfate is therefore unlikely to be related to its possible hydrolysis and reconjugation with glucuronic acid. When the total excretion of DHEA by the two groups was compared by summation of both sulfate and glucuronide conjugates, it was evident that the lower total excretion of DHEA by the essential hypertensive group was the result of the reduced excretion of the DHEA-sulfate fraction.

The total excretion of DHEA (glucuronides plus sulfates) by the normal subjects ranged from 0.42 - 7.1mg/24hr, which is comparable to the results of Vestergaard (1962, 0.4 - 9.3mg/24hr), Nowaczynski et al (1968, 0.83 - 6.4mg/24hr), and Jungman et al (1967, 0.45 - 6.1mg/24hr). Lower values have been reported by Keutman and Mason (1967), Brooksbank and Salokangas (1959), Beale et al (1971a) and Faucette and Cawley (1971). The reason for these variations is not known, but may reflect differences in methodology. In addition, considerable daily variations in the excretion of DHEA have been reported (Keutman and Mason, 1967), and more recently DHEA has been found to be secreted episodically, rather than diurnally (Saxena et al, 1970; Rosenfeld et al, 1971). The latter might be expected to affect the estimations on single blood samples, but is probably not so critical for urine that is collected over a 24 hour period.

Keutman and Mason (1967), in a detailed study of the influence of age on the excretion of 17-ketosteroids, found a progressive decline in the excretion of DHEA by subjects of both sexes aged from 20 years to over 60 years. In the present study, the number of subjects at each age was limited, but Fig. 9 illustrates that the excretion of DHEA did not decline noticeably with advancing age. The largest group consisted of hypertensive females (28) who excreted a mean of 0.70mg DHEA/24hr in the age range 39 - 69 years, whereas the normal female subjects (20) excreted a mean of 2.64mg DHEA/24hr in the age range 21 - 78 years. Keutman had the highest values for DHEA in the females aged 20 - 29 years (1.18mg/24hr) and then found a rapid decline to 0.38 (30 - 39 years), 0.21 (40 - 49 years), 0.05 (50 - 59 years) and 0.38mg/24hr (60 years). These values are considerably lower than have been found in the present study.

The reduced excretion of DHEA-sulfate by essential hypertensives appears to be rather anomalous in that the secretion rate of DHEA and DHEA-sulfate is five and six times higher respectively in benign essential hypertension (Shao et al, 1970). There could be three reasons for this:

(1) Since the circulating DHEA-sulfate is mostly bound to protein (Plager, 1965), a change in the protein binding of this compound in essential hypertension could alter its excretion rate (Shao et al, 1970). In this context, it is of interest to note that in the normal subject the biological half-life of DHEA-sulfate is considerably extended, being 8 - 11 hours for the sulfate as compared to only 30 minutes for the free compound (Baulieu et al, 1965). So far, the effect of protein binding on the half-life of DHEA-sulfate has not been investigated in essential hypertension.

(2) The second alternative is that the metabolic clearance rate of DHEA-sulfate may be altered in essential hypertension. This would seem a likely possibility, since the metabolic clearance rate of aldosterone has already been found to be reduced in this group (Nowaczynski et al, 1971; Lommer et al, 1972), but so far no study has been made with DHEA.

(3) A third alternative could be that DHEA is further metabolised prior to its excretion. This possibility was examined in the present work by comparing the excretion of two of its major metabolites, androsterone and etiocholanolone.

Excretion of androsterone and etiocholanolone

Androsterone and etiocholanolone are formed primarily by the metabolism of DHEA, though it is recognised that they are not the exclusive metabolites of this compound. They may also be derived from androstenedione, testosterone and several other minor sources such as, 5α-androstan-3,17-dione, androst-1,4-diene-3,17-dione and 21-deoxycortisol (Dorfman and Unger, 1965).

Twenty-four percent of the total 11-deoxy-17-ketosteroids comes from the testicular hormone, testosterone, of which 70% consists of androsterone and etiocholanolone (Rubin et al, 1954). The male also metabolises testosterone to androstenedione, which in turn gives rise to androsterone and etiocholanolone (Horton and Tait, 1966).

The excretion of these two compounds by normal and hypertensive subjects has been studied by estimating both the glucuronide and sulfate fractions individually. In a second group of subjects the total excretion of androsterone and etiocholanolone (G+S) was measured.

The excretion of androsterone and etiocholanolone by the normal subjects are compared with the results of other investigators in the following table. The differences observed may be due to the differences present in the methodology.

Author	Range (mg/24hr) Total Population	
	Androsterone	Etiocholanolone
This report	0.95 - 3.30	0.57 - 3.60
Beale et al (1971a)	1.00 - 4.70	1.20 - 3.00
Faucette and Cawley (1971)	0.12 - 5.00	0.20 - 6.80
Garmendia et al (1971)	0.32 - 5.70	0.09 - 1.20
Keutman and Mason (1967)	0.68 - 6.60	1.50 - 5.10
Rivera et al (1967)	1.20 - 3.80	0.87 - 2.60

Keutman and Mason (1967) and Pincus et al (1955) have found that the excretion of androsterone and etiocholanolone declines with advancing age. However, the limited number of subjects studied in this report failed

to show a similar decline. Since in our study the number of female subjects (20) predominated over the males (13), the excretion of A and E by the females in different age groups may be compared with other reports. Keutman and Mason (1967) found that females aged 20 - 29 years excreted a mean of 3.29mg/24hr of both A and E, which dropped to 1.92 and 2.93mg/24hr in the age range 30 - 39 years. A and E continued to decline to 2.04 and 2.27mg/24hr at 40 - 49 years and 0.68 and 1.52mg/24hr for those aged 60 and over. By comparison, the females studied in the present work excreted the following: 2.28 and 1.39mg/24hr of A and E respectively (20 - 29 years), 2.25 and 2.10mg/24hr (30 - 39 years), 2.12 and 1.50mg/24hr (40 - 49 years) and 2.30 and 1.70mg/24hr (60 years and over). Pincus et al (1955) have noticed a four-fold decline in the excretion of these two steroids by both sexes in the age range of 20 years to over 70 years. Although a general decline with age was concluded to occur by Keutman and Mason (1967), it can be seen from their results that several individuals over sixty years of age were exceptions to this rule in that they excreted considerably higher levels of A and E than the mean value. The actual mechanism of the effect of age on steroid excretion is not known. Both Pincus and Keutman have suggested that the lower excretion of A and E with old age may reflect a reduced production of precursors such as testosterone and androstenedione. This may be the result of a decline in the processes involved in side chain cleavage at C-17, which leads to the formation of androgenic precursors. (Pincus et al, 1955; Keutman and Mason, 1967).

The excretion of etiocholanolone glucuronide by the essential hypertensive group was found to be significantly less ($P<0.001$) than for the normal group. By contrast, the excretion of androsterone glucuronide was increased ($P<0.01$) and no differences were found in the excretion of the sulfate conjugates of this compound or etiocholanolone. The total excretion of etiocholanolone (G+S) again was reduced ($P<0.001$) in the hypertensive group mainly due to a marked reduction in the excretion of etiocholanolone

glucuronide, but no difference was found for androsterone (G+S). Since the total excretion of these two 17-ketosteroids was reduced rather than increased in the hypertensive group, it refutes the hypotheses that the reduced excretion of dehydroepiandrosterone was the result of further peripheral metabolism to these compounds.

Porportions of 5α/5β-reduced metabolites

Comparison of the levels of androsterone and etiocholanolone excreted by the normal and essential hypertensive subjects gives an indication of the steroid-5-reductase activities involved in the formation of these compounds. Since the normal group excreted more etiocholanolone than androsterone in the glucuronide fraction (ratio 3:1); this suggested that the 5β-reductase activity was greater leading to the excretion of more glucuronides. By contrast, the ratio of etiocholanolone to androsterone in the sulfate fraction was 1:4, suggesting that 5α-reductase activity is greater, and gives more sulfate conjugates.

The ratio of androsterone over etiocholanolone gives a measure of the relative 5α/5β-reductase activities. This ratio was markedly reduced among the essential hypertensives probably due to the significantly reduced excretion of etiocholanolone glucuronide, which suggests that there is a relative deficiency of 5β-reductase activity in this group. The generally decreased 5-reductase activity which has been found for the metabolism of corticosteroids in essential hypertension by Kornal and Takeda (1967) will be discussed later, but it is apparently not confined to either the 5α- or the 5β-reductase activity specifically.

The interpretation of the significance of the differences in the excretion of androsterone and etiocholanolone and, the androsterone/etiocholanolone ratio in essential hypertension, must be tempered with caution since it

is recognized that several factors can alter these values. Administration of triiodothyronine (Bradlow et al, 1956) and thyrotoxicosis (Peterson, 1958) favours the production of androsterone over etiocholanolone. In addition, alterations in the excretion of both androsterone and etiocholanolone have been found in several forms of cancer which include myelogenous leukemia (Gallagher et al, 1962), cancer of the breast (Bullbrook et al, 1960, 1962, 1970; Atkins et al, 1964; Marmorston et al, 1965), prostate (Gallagher et al, 1963; Marmorston et al, 1965), and lung (Marmorston et al, 1965). Whether these alterations in the peripheral metabolism of 17-ketosteroids are direct consequences of the specific disease or are "non specific consequences of illness" as has been suggested by Zumoff et al (1971) is debatable. Obviously the different medications received by the patients in these diverse studies may influence the metabolism of the 17-ketosteroids and this makes the drawing of conclusions hazardous. However, the essential hypertensive subjects investigated in the present study were free from medications and the differences detected may be related more directly to the effects of the elevated blood pressure.

Excretion of androstenedione

Since it was found that the essential hypertensives excreted significantly low levels of the 5β- reduced compound, etiocholanolone, it was of interest to determine whether the excretion of one of its precursors, androstenedione, was also altered. This compound is a "Δ^4-3-keto" steroid, which is both an intermediate in the biosynthesis of androgens and a metabolite of dehydroepiandrosterone. Its metabolism is rather complicated, but basically involves reduction of the Δ^4-double bond and the ketonic groups at C-3 and C-17. Dorfman and Unger (1965) have listed 33 possible compounds that may arise from androstenedione; however, two major ones are androsterone and etiocholanolone.

In the present study, the excretion of free androstenedione was measured in the urine of 13 normal (3 males, 10 females) and 15 hypertensive (4 males, 11 females) subjects. Since the number of male subjects studied was limited, no attempt was made to divide the results between the sexes. The normal subjects excreted 0.23 - 5.9ug of androstenedione per 24hr urine in the total population.

There have been relatively few reports on the excretion of androstenedione in the urine of normal subjects (Tajic, 1965; Longhino et al, 1968; Schollberg and Hubl, 1968; Schollberg et al, 1969; Hubl et al, 1971). Considerable higher values than those reported here have been found for both females (5 - 52.8ug/24hr, Schollberg and Hubl, 1968; 1.1 - 11.4ug/24hr, Tajic, 1965); and males (6 - 65ug/24hr, Hubl et al, 1971; 6.0 - 18.3ug/24hr, Tajic, 1965). The reason for the markedly lower values obtained in the present study is not clear, but may be related to the levels of free androstenedione estimated by us as compared to the "total" urinary levels measured by others. Thus, Tajic (1965), Schollberg (1968) and Hubl (1971) incubated the urine with β-glucuronidase in order to hydrolyse any enol-glucuronide conjugates of androstenedione (Wotiz, 1962) that may be present. However, the contribution of the enol-glucuronide type conjugate of androstenedione has not been fully evaluated. A single report found that the concentration of the enol-glucuronide was less than 5ug per 24hr (Schubert, 1958). The higher levels of androstenedione reported by these workers is more likely to be related to differences in methodology; primarily the use of fluorometric methods which are considerably less specific than the GLC procedures used in the present study.

In the present study, the normal group excreted a mean of 2.26ug of androstenedione per 24 hours which is lower than that excreted by the hypertensive group (4.16ug/24hr). The difference was significant at the $P<0.001$ level.

It is tempting to speculate that the increased excretion of androstenedione by the hypertensive group may reflect the reduced 5β-reductase activity which has already been noted for this group of subjects.

Excretion of 11-oxygenated-17-ketosteroids

Effects of substituents of the steroid nucleus on ring-A reduction

The presence of an "oxy" function at C-11 of the steroid nucleus has been shown to influence the activity of steroid-5-reductase enzyme systems (Brown-Grant et al, 1962; Dorfman and Unger, 1965). It orients the reduction of the double bond between C-4 and C-5, so that the majority of the metabolites are 5α- reduced compounds. Two of the major ring A-unsaturated steroids, namely 11β-hydroxyandrostenedione and androst-4-en-3,11-17-trione (adrenosterone), are metabolised primarily to the 5α- reduced compounds, namely, 11β-hydroxyandrosterone and 11-ketoandrosterone, with only 20% of the metabolites having the 5β- configuration (Dorfman and Unger, 1965; Cope, 1972).

The presence of the 17β-corticosteroid side chain of cortisol and cortisone directs the formation of 5β- reduced compounds and yields primarily 11β-hydroxyetiocholanolone and 11-ketoetiocholanolone after side chain cleavage. Whereas metabolism of the corticosteroids produces four times more 5β- than 5α- metabolites (Cope, 1972), the total contribution of these compounds to the 11-oxygenated-17-ketosteroids is relatively small. Following administration of cortisol to humans, only 2% is converted to 11-oxy-17-ketosteroids (Gallagher, 1954; Bradlow and Gallagher, 1957; Dorfman and Unger, 1965), and recently the conversion of 3α, 11β, 17α, 21-tetrahydroxy-5β-pregnan-20-one (allo-3α-tetrahydrocortisol) into 11β-hydroxyandrosterone by humans was found to be less than 7%. This indicates that this compound does not contribute significantly to formation of the 11-oxy-17-ketosteroid fraction (Goldzieher and Axelrod, 1971).

The effect of these ring substituents on the relative proportions of 5α- and 5β- reduced metabolites was examined in the present study by measuring the levels of 11-ketoandrosterone, 11β-hydroxyandrosterone, 11-ketoetiocholanolone and 11β-hydroxyetiocholanolone in the $C_{19}O_3$ fraction. In addition, the three major 11-deoxy-17-ketosteroids, i.e. androsterone, etiocholanolone and dehydroepiandrosterone were analysed simultaneously in the 11-deoxy plus 11-oxy-17-ketosteroid fraction, since it was found that these seven compounds could be adequately resolved on a 2% XE-60 column (Fig. 5). The methodology of GLC analysis developed incorporates the procedures of several literature reports (Roberts et al, 1968; Faucette and Cawley, 1971; Matthijssen and Goldzieher, 1971). The metabolites were purified and resolved into three major groups, namely, 11-deoxy-17-ketosteroids, 11-oxy-17-ketosteroids and corticosteroids, prior to GLC analysis, by chromatography on silica gel columns. This was preferred to previous procedures that involved either thin layer or paper chromatography. As indicated above the 11-deoxy and 11-oxy-17-ketosteroid fractions separated by silica gel chromatography were recombined for GLC analysis when it was determined that the components could be effectively resolved. ^{3}H-11β-Hydroxyetiocholanolone was prepared by oxidation of ^{3}H-tetrahydrocortisol and was used to correct for losses incurred during the procedure. ^{14}C-Dehydroepiandrosterone was used as the radioactive tracer to calculate the recovery of the 11-deoxy-17-ketosteroids.

Excretion of 11-keto-and 11β-hydroxy-androsterone and etiocholanolone

The excretion of 11-keto- and 11β-hydroxyandrosterone, and 11-keto- and 11β-hydroxyetiocholanolone by the normal subjects in the present study is compared in the following table with the results of other workers. Differences in methodology are evident and may account for the variations found in the mean excretion of individual steroids.

Author and Method	Mean excretion, mg/24hr (Total Population)			
	KA	KE	HA	HE
(1) This report: Amberlite, glusulase, silica gel and GLC as TMSE	0.65	0.68	1.03	0.68
(2) Faucette and Cawley (1971): Amberlite, glusulase and GLC as TMSE	0.35	0.65	1.20	0.95
(3) Beale et al (1971): Free removed, Ketodase, solvolysis, and GLC as formates	0.07	0.73	0.63	0.65
(4) Roberts et al (1968): Glusulase, paper chromatography and colorimetry	0.10	1.18	0.61	0.34
(5) Keutman and Mason (1967): Ketodase, solvolysis, and GLC as TMSE	-	0.76	0.86	0.78
(6) Vestergaard (1962): Ketodase, simultaneous acid hydrolysis and benzene extraction, gradient elution chromatography and colorimetry	0.51	1.14	1.41	0.90
(7) Brooksbank and Salokangas (1959): Ketodase, solvolysis, silica gel and colorimetry	0.14	0.83	0.94	0.68

No decline in the urinary excretion of the 11-oxygenated 17-ketosteroids occurred with advancing age, which is in agreement with the report of Keutman and Mason (1967). The latter workers (Keutman and Mason, 1967) together with Pincus (1955) have suggested that the capacity for corticosteroid production and reduction is continued into advancing age, which contrasts with their findings for the 11-deoxy-17-ketosteroids.

Proportions of 5α/5β- reduced metabolites

The hypertensive subjects excreted more 5α- reduced compounds (3.46mg/24hr) than the normal subjects (1.68mg/24hr) and this resulted in a significant ($P<0.01$) elevation in the ratio of 5α/5β- reduced compounds. Within the hypertensive group the excretion of 5α- reduced compounds (3.46mg/24hr) was significantly higher than the 5β- reduced compounds

(1.79mg/24hr), whereas within the normal group the slightly increased excretion of 5α- compounds was not significant. These findings may indicate that in the formation of the $C_{19}O_3$-17-ketosteroids in essential hypertension, the influence of a 5α- directing "11-oxy" function is more pronounced because of a relative deficiency of 5β-reductase activity already noticed in the $C_{19}O_2$-fraction.

It was originally suggested by Dorfman that the measurement of urinary 17-ketosteroids would reflect the intensity of the production of their androgenic precursors (Rubin et al, 1954). Subsequently, the ratio of 11β-hydroxy plus 11-ketoandrosterone to 11β-hydroxy-plus 11-keto-etiocholanolone (HA+KA/HE+KE) was used as an index of the proportion of androgen to cortisol precursors produced by the adrenal in the clinical assessment of hirsutism (Bush and Mahesh, 1959). However, these conclusions have recently been reviewed extensively by Cope who bears the view that the urinary 17-ketosteroids are derived from several different physiological precursors and their levels fluctuate from time to time. For this reason it is difficult to draw clinically valuable conclusions from the urinary analysis of individual 17-ketosteroids and the information obtained is relatively limited (Cope, 1972).

The estimation of the 11-oxygenated 17-ketosteroids excreted by essential hypertensives was of interest in view of the relatively reduced 5β-reductase activity found from an examination of the proportions of 5α- and 5β- reduced 11-deoxy 17-ketosteroids. However, in the formation of 11-oxygenated 17-ketosteroids it may be concluded that the essential hypertensive group excreted significantly more 5α- reduced metabolites than the normal group. In addition, within the normal and hypertensive groups the excretion of 5α- reduced compounds was higher than the 5β- reduced compounds, although the differences were only significant in the hypertensive group.

Excretion of 17-ketogenic steroids

The excretion of 17-hydroxycorticosteroids by essential hypertensives and normal subjects was evaluated indirectly by converting these compounds to 17-ketogenic steroids. This required side chain cleavage by reduction with sodium borohydride followed by oxidation with sodium periodate. During this reaction the 11-keto group was converted to an 11β-hydroxy. Since the corticosteroids were isolated by silica gel chromatography before reaction, they were essentially free of other 17-ketosteroids. The three major 17-ketosteroids derived from the corticosteroids were the 11-deoxy compound, etiocholanolone, and the 11-oxygenated compounds, 11β-hydroxy androsterone and 11β-hydroxy etiocholanolone.

Etiocholanolone has two potential precursors, namely pregnanetriol and tetrahydro 11-deoxy-cortisol. Allo-tetrahydro cortisone, allo-cortol and allo-cortolone are the precursors of 11β-hydroxy androsterone, and tetrahydrocortisol, tetrahydrocortisone, cortol and cortolone are the precursors of 11β-hydroxy etiocholanolone. There was no evidence in the gas chromatograms from normal and hypertensive subjects for the presence of the 5β-reduced compound, androsterone. This compound would have been derived from allo-pregnanetriol and allo-tetrahydro-11-deoxy cortisol. This is in agreement with previous reports that the excretion of these two metabolites by humans is not significant (Rubin and Dorfman, 1954; Edwards et al, 1964; Dorfman and Unger, 1965; Makin, 1970).

Excretion of the 17-ketosteroids derived from the 17-ketogenic steroids

The excretion of the derived 17-ketosteroids, etiocholanolone (E), 11β-hydroxy androsterone (HA), and 11β-hydroxy etiocholanolone (HE) by the normal subjects studied in this respect is compared with other workers in the following table:

Author.	Range - mg/24hr (Total Population)		
	E	HA	HE
This report	0.41 - 2.58	0.64 - 3.30	0.61 - 5.20
Menini and Norymberski, 1965	0.30 - 2.14	0.33 - 3.53	2.10 - 7.90
Layne et al, 1962) Romanoff et al, 1961)	-	0.30 - 1.40	4.80 -10.10
Fotherby and Love, 1960	0.90 - 1.40	-	-

Here again, methodological differences are present between the investigators which may be the reason for the variations found.

In the hypertensive group, the excretion of the derived etiocholanolone (1.57mg/24hr) was slightly above that of the normal subjects (1.06mg/24hr), but the difference was not significant. On the other hand, the excretion of derived 11β-hydroxyandrosterone (3.22mg/24hr) and 11β-hydroxyetiocholanolone (3.97mg/24hr) was significantly increased in the hypertensive group. As a result of these increases the ratio of the 5α/5β- reduced compounds was not altered. It may be of interest to compare these results with those given in the paper of Kornel et al (1969) on corticosteroid metabolism in essential hypertension where the metabolites were separated into several fractions and analysed. In this paper ring-A unsaturated compounds were conspicuous in the urine of the hypertensive subjects. Thus, cortisol and corticosterone predominated in the free steroid fraction while 6α- and 6β- hydroxy cortisols were elevated in the sulfate fraction. By contrast, ring-A reduced compounds excreted as glucuronide conjugates, namely tetrahydro reduced metabolites of cortisol, cortisone, 11-dehydrocorticosterone and corticosterone were quantitatively less important in the urine of the hypertensives as compared to the normal subjects.

These findings led Kornel to postulate that essential hypertensives have a characteristic defect in the steroid ring-A reducing systems (Kornel et al, 1969). He did not, however, indicate whether this was more pronounced for the 5α- or 5β- reducing system. Kornel's methodology also differed from the present report in several ways. Notably, several metabolites: namely, pregnanetriol, cortols and cortolones, were excluded but were measured as 17-ketogenic steroids in the present study. The contribution of pregnanetriol is significant since normal subjects excrete 0.93 - 2.0mg/ per 24hr. (Genest et al, 1960; Rivera et al, 1967), and cortols and cortolones contribute a further 7 - 10% and 20 - 25% respectively to the total corticosteroid fraction (Fukushima et al, 1960; Romanoff et al, 1961; Besch and Barry, 1964; Beale et al, 1971b).

In Kornel's report, the total excretion of corticosteroids (Porter-Silber chromogens) by the hypertensives (14.8mg/24hr) was higher than the normal subjects (12.25mg/24hr), but the difference was not significant ($P<0.1$). In the present report the excretion of 17-ketogenic steroids, as estimated by GLC, was also higher in the hypertensive group (mean 8.78mg/24hr) as compared to a mean of 5.28mg/24hr excreted by the normal subjects. In this case the difference was statistically significant ($P<0.001$).

Proportions of 5α- and 5β- reduced 17-ketogenic metabolites

In the previous section it was noted that during the metabolism of corticosteroids, the C-17- side chain primarily orientates the reduction of the Δ^4-double bond to give 5β- reduced compounds (Brown-Grant et al, 1962; Dorfman and Unger, 1965; Cope, 1972).

It was evident from the present study that although the formation of the derived 5α- and 5β- reduced metabolites (11-oxygenated) was relatively greater in the hypertensive group than the normal subjects,

there was no significant difference in the ratio of these 5α/5β- reduced compounds between the two groups. However, the total excretion of the derived 5β- compounds, etiocholanolone plus 11β-hydroxyetiocholanolone, was higher than the major 5α- compound, 11β-hydroxyandrosterone in both normal and hypertensive groups. Also the excretion of the total 17-ketogenic steroids, E+HA+HE, was significantly higher in the hypertensive group compared to the normal group. These findings may reflect the influence of both an "11-oxygen" function and the C-17 corticosteroid side chain, but the overall influence of the side chain appears to be greater.

11-oxygenation Index

From the measurement of the 11-deoxy and 11-oxy-17-ketogenic steroids it is possible to derive a potentially useful factor, the "11-oxygenation index", which is the ratio of the 11-deoxy-17-ketosteroids/11-oxygenated-17-ketosteroids derived from the corticosteroids. The 11-oxygenation index may indicate the presence of an 11β-hydroxylase enzyme deficiency, which would primarily result in the over production of 11-deoxy-cortisol. The "index" has been of value in the diagnosis of congenital adrenal hyperplasia, which is an inborn metabolic abnormality involving 11β-hydroxylase deficiency in one of its forms. This blocks the biosynthesis of cortisol and other 11-oxy-corticosteroids (Hill, 1960; Edwards et al, 1964; Bondy, 1969; Makin, 1970).

In our study, the "index" for the normal subjects ranged from 0.11 - 0.37 while the hypertensives had 0.08 - 0.36. The "index" for the normal subjects found by others were in the ranges of (0.06 - 0.57, Hill, 1960), (0.13 - 0.62, Edwards et al, 1964), (0.09 - 0.6, Makin, 1970). It may therefore be concluded that 11β-hydroxylase activity is normal in the essential hypertensives.

Evaluation of the colorimetric assay of 17-ketosteroids, 17-ketogenic steroids and 17-hydroxycorticosteroids

It has been noted that the group estimations of 17-ketosteroids, 17-ketogenic steroids, and 17-hydroxycorticosteroids do not correlate well with the values obtained by the summation of the gas liquid chromatographic results. In the following table, colorimetric and GLC values are compared in the normal and hypertensive groups.

Steroid determination	Mean excretion (mg/24hr)			
	Normal		Hypertensive	
	Color	GLC	Color	GLC
Total 17-ketosteroids (A+E+DHEA)	7.11	7.21	5.91	4.28
Total 17-ketosteroids A+E+DHEA+KA+HA+KE+HE)	6.97	7.99	4.58	9.27
Total 17-ketogenic steroids	9.36	5.28	4.72	8.78
Total 17-hydroxycorticosteroids	5.07	-	2.76	-

There may be several reasons for these discrepancies. In general, 77% and 83% of the total urinary 17-ketosteroids in the males and females respectively, comes from androsterone, etiocholanolone, dehydroepiandrosterone, 11-ketoandrosterone and etiocholanolone, and 11β-hydroxyandrosterone and etiocholanolone (Cope, 1972). During colorimetric assay of total 17-ketosteroids, hot acid hydrolysis causes partial destruction of dehydroepiandrosterone, 11β-hydroxyandrosterone and etiocholanolone. This treatment is also dependent on pH, temperature and duration of hydrolysis (Lieberman et al, 1954; Munson and Kenny, 1954; Cawley et al, 1965; Metcalf, 1971).

The presence of carbohydrate compounds in the urine interferes with the formation of the Zimmerman chromogens and produces non-specific

chromogens. This reaction is also dependent on the concentration of alkali, temperature and duration of incubation (Ernest et al, 1964; Keutman and Mason, 1967). The proportion of non-specific chromogens formed could be as high as 80%, if the experimental conditions are not controlled properly (Goldzieher and Axelrod, 1962). However, this problem is corrected to a great extent by extraction of the chromogens with organic solvents (Peterson and Pierce, 1960) and by application of a color-correction formula (Allen, 1950).

In the determination of Porter-Silber chromogens, it has been found that 20 - 25% of cortisol, cortisone and tetrahydrocortisone, and 40 - 45% of tetrahydrocortisol suffered loss (Peterson et al, 1957; Cope, 1972). Vermeulen (1957) and Ernest et al (1964) have accounted for 40 - 50% and 76% respectively, of the total urinary 17-hydroxycorticosteroids by the colorimetric method. Here again, application of color-correction formula has improved the estimations greatly (Hertoghe et al, 1955).

In spite of all these drawbacks, however, the colorimetric assay procedures are still widely used in routine estimations (Vermeulen, 1957; Muehlbacher and Smith, 1970).

Steroid 5- reductases and the possible influence of elevated blood pressure.

The differences that have been found in the proportions of 5α- and 5β- reduced metabolites excreted by normal and hypertensive subjects have so far been considered in relation to the effects of different substituents in the steroid ring systems. Thus, an "11-oxygen" function directs reduction at C-5 to give primarily 5α- reduced metabolites, and a "corticosteroid side chain" favours the formation of primarily 5β- reduced metabolites (Brown-Grant et al, 1962; Dorfman and Unger, 1965; Cope, 1972). This infers that the 5- reductase enzyme activities will vary according to the type of

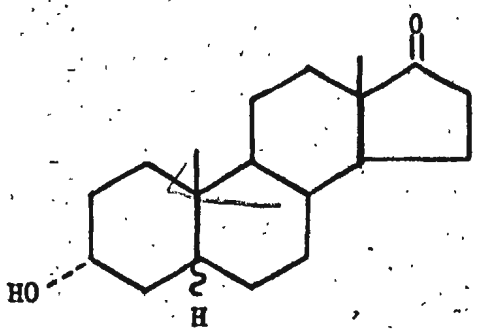
substrate. Early studies have indicated that a 5α- reductase is located in the microsomal fraction and a 5β- reductase in the soluble fraction of the liver (Forchielli and Dorfman, 1956; Brown-Grant et al, 1962; Van Doorn et al, 1973).

Differences in the excretion of 5α- and 5β- reduced metabolites by the normal and hypertensive subjects studied in the present work may best be explained by considering that there are several 5α- and 5β- reductase enzyme systems that are specific for different substrates. Several reports support the concept of substrate specific steroid-reductase enzyme systems in the liver (Tomkins et al, 1957; McGuire and Tomkins, 1960; Brown-Grant et al, 1962; Briggs and Brotherton, 1970; Bock, 1971).

It can be seen from the following diagram that there is a trend in the relative proportions of 5α- and 5β- reduced metabolites from the 11-deoxy-17-ketosteroids through the 11-oxy-17-ketosteroids to the 17-ketogenic steroids (derived from the corticosteroids).

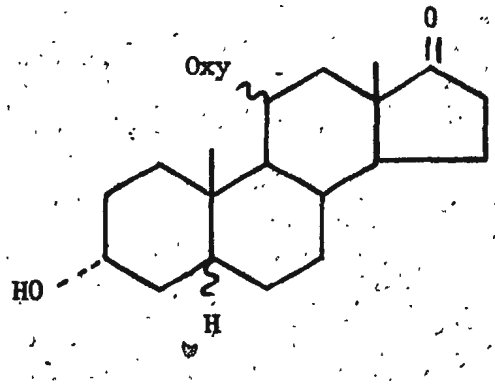
The hypertensive subjects excreted significantly less 5β-11-doexy-17-ketosteroids than the normal subjects, and it was suggested that the hypertensives had a relative 5β- reductase deficiency. However, when the proportions of 5β- and 5α- reduced 11-oxygenated-17-ketosteroids were compared, it was evident that the 5β- reductase enzyme system was as active in the hypertensive group as for the normal group. The increased proportion of 5α-reduced metabolites may be explained by the predominantly 5α- directing effect of the "11-oxygen" function. But in order to explain the "increased" 5β-reductase activity by the hypertensives for the 11-oxygenated-17-ketosteroids as compared to the 11-deoxy-17-ketosteroids, it may be necessary to hypothesise either (a) the presence of one or more specific 5β- reductase enzyme systems, one for the 11-deoxy-, the other for the 11-oxy-17-ketosteroids, or

A. $C_{19}O_2$- (11-deoxy)-17-ketosteroids



5α →
5β ↓

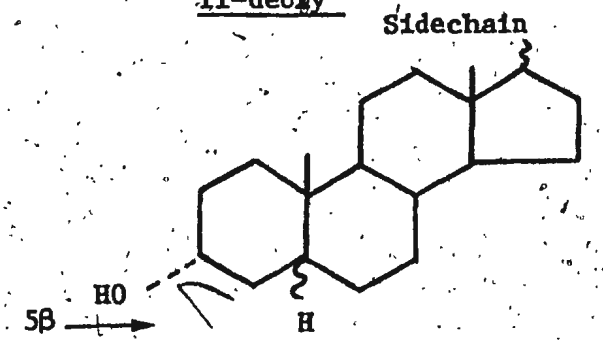
B. $C_{19}O_3$- (11-oxygenated)-17-ketosteroids



5α ↑
5β →
OXY = Keto or Hydroxy

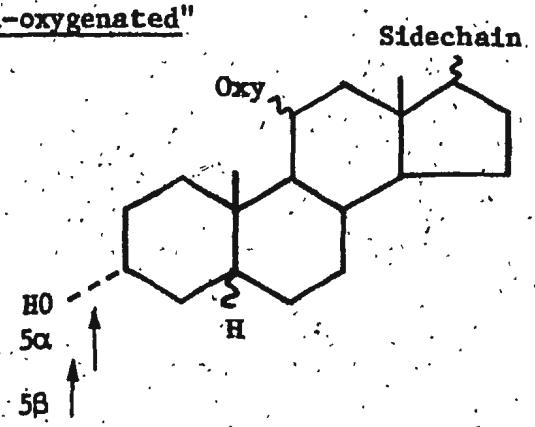
C. Corticosteroids (17-ketogenic steroids)

"11-deoxy"



5α Not detectable

"11-oxygenated"



OXY = Keto or Hydroxy

(b) the reduced activity of a single 5β- reductase enzyme system towards the 11-deoxy-17KS as compared to the 11-oxy-17-ketosteroids.

Again, for the 17-ketogenic steroids derived from the corticosteroids, the 5β- directing effect of the "17β- side chain" was noted. It may be speculated that the markedly increased excretion of both 5α- and 5β- reduced metabolites by the hypertensive subjects may be the result of: (a) different 5- reductase enzyme systems for this group of steroids, or (b) greater 5- reductase enzyme activity in the hypertensive group due to increased sensitivity to the directing effect of the 11-oxy and corticosteroid side chain substituents.

That an elevation in blood pressure may affect the activity of certain enzyme syatems has been suggested by several studies on the hepatic metabolism of corticoids by the hypertensive rat. (Silah, 1970; Turcotte and Silah, 1970; Silah, 1971). Ultimately, one has to relate the differences that have been found in the proportions of reduced steroid metabolites, and presumably differences in enzyme activities, in the hypertensive group, to the influence of the elevated blood pressure. So far evidence for these effects has been indirect, but future studies may reveal a more direct cause-effect relationship between elevated blood pressure and the formation of different metabolites.

FUTURE STUDIES

The results given in this thesis suggest that elevated blood pressure may alter the enzyme activity such as Δ^4-5-reductases, involved in the metabolism of steroids. Future efforts may be directed towards understanding whether elevated blood pressure has a direct effect on steroid metabolism and how it exerts such effect. Studies on essential hypertension before, during and after treatment with anti-hypertensive agents, could indicate whether certain steroid enzyme activities alter under the influence of blood pressure.

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Publications arising from this work

1. Dey, A.C., Abbott, E.C., Rusted, I.E. and Senciall, I.R. (1972). Excretion of conjugated 11-deoxy-17-ketosteroids in Essential Hypertension. Can. J. Biochem., 50, 1273-1281.

2. Dey, A.C. and Senciall, I.R. (1973). Urinary excretion of 11-oxygenated 17-ketosteroids in Essential Hypertension. Procd. Can. Fed. Biol. Soc., 16, 53, (212). (Abstract).

3. Dey, A.C. and Senciall, I.R. (1974). Influence of elevated blood pressure and steroid substituents on the excretion of ring-A reduced metabolites by females. Procd. Can. Fed. Biol. Soc., Submitted. (Abstract).

4. Dey, A.C. and Senciall, I.R. (1974). Influence of elevated blood pressure and steroid substituents on the excretion of ring-A reduced metabolites by females. In preparation for "Steroids".